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Title of Thesis: Stimulation-Induced Changes in Frog Neuromuscular Junctions. A Quantitative Ultrastructural Comparison of Rapid-Frozen and Chemically Fixed Nerve Terminals.

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ABSTRACT

Title of Dissertation: Stimulation-induced changes in frog neuromuscular junctions. A quantitative ultrastructural comparison of rapid-frozen and chemically fixed nerve terminals.

Patricia Anne Brewer, Doctor of Philosophy, 1984

Dissertation directed by: Kathryn J. Lynch, Ph.D.
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This study was conducted to determine the presynaptic morphological changes due to neural activity in rapidly stimulated neuromuscular junctions preserved by rapid-freezing and freeze-substitution and to ascertain what, if any, additional changes are produced by chemical fixation.

Cutaneous pectoris nerve-muscle preparations from juvenile Rana pipiens were rapid-frozen against a copper block cooled to $8^{\circ}K$ and freeze-substituted with OsO_4 in acetone. This frozen material was compared to material which had been chemically fixed by immersion in glutaraldehyde and post-fixed in OsO_4/ferrocyanide.

The nerves of experimental preparations were stimulated at 15 Hz for 15 minutes with a suction electrode and then either fixed or frozen one minute after cessation of the stimulus. Rested preparations were fixed

or frozen 60 minutes after cessation of the stimulating current. Control preparations were unstimulated and preserved either by chemical fixation or rapid-freezing.

This study provides evidence that most of the previously reported effects of stimulation are real; that is, they are present in rapid-frozen fresh (unfixed) tissue. However, there were significant differences in the reactions of the tissue to the two types of preservation.

Rapid-freezing confirmed that rapid stimulation causes a reduction in the number of synaptic vesicles and an increase in the number of cisternal profiles, changes reported by previous investigators in chemically fixed tissue. The rapid-frozen preparations in the present study showed, in addition, that rapid stimulation produces an increase in synaptic vesicle diameter, a change in the shape of mitochondria without any detectable change in their volume and a decrease in the number of microtubules. Terminals preserved by either method exhibited recovery toward control morphology with 60 minutes of rest after stimulation.

Differences between the frozen and fixed groups indicated the effects of chemical fixation on nerve terminals. Fixation appeared to cause general swelling of the tissue; the terminals and most organelles were larger in the fixed material. Some presynaptic elements, most

notably the nerve terminals and their mitochondria were even more susceptible to swelling after stimulation. Differences in cisternal size and number suggested that fixation causes cisternal fusion.

In nerve terminals soaked or stimulated in high calcium Ringer's solution and preserved by rapid-freezing the reduction of the number of synaptic vesicles was more profound than in terminals soaked or stimulated in normal Ringer's solution. Synaptic vesicle diameter, however, was not noticably affected by the change in calcium concentration. Large increases in the number of cisternae per terminal suggested that increased membrane recycling was in progress.

Nerve terminals stimulated in a Ringer's solution where calcium had been replaced by magnesium exhibited a reduction in synaptic vesicle number but no mitochondrial changes or increase in cisternal numbers. Synaptic vesicle diameter, however, increased to its highest level in the terminals stimulated in high magnesium.

Preparations were also soaked and stimulated in hemicholinium-3 (HC-3), a blocker of choline uptake, to determine if acetylcholine reloading played a role in the stimulation-associated increase in synaptic vesicle size. HC-3 appeared to be an irritant of the preparation; soaking alone produced the stimulation-associated constellation of changes, except for increased synaptic

vesicle diameter. Stimulation and a subsequent period of rest enhanced these changes.

STIMULATION-INDUCED CHANGES IN FROG NEUROMUSCULAR JUNCTIONS. A QUANTITATIVE ULTRASTRUCTURAL COMPARISON OF RAPID-FROZEN AND CHEMICALLY FIXED NERVE TERMINALS

by

Patricia Anne Brewer

Dissertation submitted to the Faculty
of the Department of Anatomy Graduate Program of the
Uniformed Services University of the Health Sciences
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for the degree of
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For my family

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INTRODUCTION

I. Neuromuscular junction morphology

The term "synapse", first used by Sir Charles Sherrington (Foster and Sherrington, 1897), signifies the site where two neurons come into functional contact with each other. This description can be expanded to include not only functional contacts between neurons but also between neurons and effector cells such as muscle fibers. Most of the morphological and physiological information first collected about synapses was through studies of the neuromuscular junction (NMJ).

The first report of NMJ ultrastructure was made in 1954 by Palade. He described a nerve terminal specialization containing mitochondria and small (30-50 nm) vesicles. Extensive morphological studies have corroborated this early description (including those by DeRobertis and Bennett, 1955; Robertson, 1956; Birks, Huxley and Katz, 1960). Other investigators have described similar presynaptic specializations in the central nervous system (eg. Gray, 1963). The small, ubiquitous structures found by all of these authors were designated "synaptic vesicles" (DeRobertis and Bennett, 1955) and were postulated to be "droplets" of

pharmacologically active substances.

The current description of the NMJ is more complete. As a motor nerve axon approaches its muscle fiber, it loses its myelin sheath and divides into terminal branches which form the NMJ. The terminals contain numerous mitochondria, tubules of smooth endoplasmic reticulum (SER), cytoskeletal elements (microtubules and neurofilaments), occasional dense-cored vesicles (DCVs) and thousands of synaptic vesicles. Each terminal lies in a depression in the surface of the muscle fiber, the "synaptic gutter". In this region the muscle membrane is thrown into junctional folds. At the crests of the folds the postsynaptic membrane is separated from the presynaptic membrane by a synaptic cleft of about 60 nm. The cleft is lined by a basal lamina which follows the infoldings of the muscle sarcolemma. Within the nerve terminals, synaptic vesicles cluster together near specialized areas of the terminal plasmalemma, overlying the openings of the junctional folds. These specialized presynaptic areas have been termed "active zones" (Couteaux and Pecot-Dechavassine, 1970). Dreyer, Peper, Akert, Sandri and Moor (1973) showed that in frog NMJs, active zones are 100 nm wide bands oriented perpendicular to the long axis of the nerve terminal and possessing a density on their cytoplasmic aspect. Although the length and orientation of the zones vary with animal, active

zones are a consistent feature of NMJs (Ellisman, Rash, Staehelin and Porter, 1976; Walrond and Reese, 1981).

II. Physiology of the neuromuscular junction

At the time when the ultrastructure of the NMJ was first being described, discoveries were being reported in the physiology of that system that correlated well with its structure. Using intracellular recording techniques, Fatt and Katz (1951) characterized the electrical properties of frog motor endplates. When they stimulated the nerve, they recorded a transient change in the electrical potential across the muscle membrane at the NMJ. They called this the endplate potential (EPP) and felt that it was caused by the release of acetylcholine (ACH), the transmitter at the NMJ (Dale, Feldberg and Vogt, 1936). Later these same authors observed spontaneous electrical depolarizations at the frog NMJ and called them miniature endplate potentials (MEPPs) (Fatt and Katz, 1952). DelCastillo and Katz (1954a) showed that MEPPs were due to the spontaneous release of uniform quanta of transmitter. They also demonstrated that EPPs are integral multiples of a unit of the same amplitude as the MEPPs and postulated that they are from the same source (DelCastillo and Katz, 1954a, 1954b).

III. Presynaptic events during neuromuscular transmission: vesicular release

A. The vesicle hypothesis

The physiological evidence for quantal release and the presence of synaptic vesicles of small uniform size resulted in the "vesicle hypothesis", first proposed by Robertson in 1956. According to this hypothesis, the contents of the synaptic vesicles correspond to the quanta of neurotransmitter detected electrophysiologically. Although there are still some experimental findings that appear to refute this (Wernig and Stirner, 1977; Tauc, 1979; Dunant, 1981), a great deal of evidence supports the vesicle hypothesis (eg. Zimmerman and Whittaker, 1974; Zimmerman and Denston, 1977a, 1977b; Heuser, Reese, Dennis, Jan, Jan and Evans, 1979; Zimmerman, 1979).

A combination of many physiological, anatomical and biochemical studies has produced the following theory of neurotransmission at the NMJ: Upon depolarization of the presynaptic terminal membrane by a nerve action potential, calcium ions rush into the terminal from the extracellular space. The increase in intracellular calcium concentration causes the synaptic vesicles closest to the membrane to fuse with the membrane and discharge their contents. The transmitter diffuses across the

synaptic cleft (taking approximately 50 μsec; Shepard, 1969), to bind to ACH receptors on the postsynaptic muscle membrane. This produces a transient increase in the permeability of that membrane to ions, resulting in a local transient depolarization, the EPP. If the EPP exceeds a threshold value, a self-propagating potential change, an action potential, is initiated in the surrounding muscle membrane. The action potential then initiates muscle contraction. (For review see Lester, 1977.)

If the synaptic vesicles release their contents into the synaptic cleft as proposed, there should be some morphological evidence for contact between synaptic vesicles and the plasma membrane of the terminal. Some authors originally believed that neurotransmitter was released into the synaptic cleft by a momentary contact between the vesicle and the presynaptic membrane (Katz, 1958, 1962; Pfenninger, Akert, Moor and Sandri, 1971, 1972). The use of freeze-fracture techniques showed that a more extensive interaction between the membranes was likely. It was proposed that dimples seen in freeze-fractured presynaptic membranes in the central nervous system (CNS) corresponded to permanent attachment sites where vesicles attached to the membrane, and, upon stimulation, released their contents into the synaptic cleft (Streit, Akert, Sandri, Livingston and Moor, 1972;

Pfenninger and Rovainen, 1974). Openings in the fused membranes were thought to be temporary, present only during synaptic activity. Transmission electron microscopy revealed omega-shaped deformations in the presynaptic membrane of NMJs (Birks, Huxley and Katz, 1960; Hubbard and Kwanbunbumpen, 1968; Couteaux and Pecot-Dechavassine, 1970). Like the "open" dimples seen in freeze-fractured CNS synapses, these omega figures were thought to represent vesicles suspended in a state of fusion with the plasma membrane.

Subsequent freeze-fracture work revealed surface deformations in presynaptic membranes adjacent to the linear active zones at frog NMJs (Dreyer, Peper, Akert, Sandri and Moor, 1973; Peper, Dreyer, Sandri, Akert and Moor, 1974; Heuser, Reese and Landis, 1974; Ceccarelli, Grohavaz, Hurlbut and Iezzi, 1979a, 1979b). Heuser, Reese and Landis (1974) found that these deformations were present, however, only in synapses active at the time of fixation. No permanent attachment sites could be verified. Because the dimples had a variety of sizes and shapes, these investigators postulated that these definitions represented synaptic vesicles in various stages of discharge. The most direct evidence for the vesicle hypothesis is from work by Heuser, Reese, Dennis, Jan, Jan and Evans (1979). Using varying concentrations of 4-aminopyridine (4-AP) to increase the number of

transmitter quanta discharged per nerve impulse, they showed (in unfixed, rapid-frozen, freeze-fractured tissue) that the number of exocytotic vesicles caught by rapid-freezing increased commensurately. With statistical analysis they were able to show that an individual vesicle fuses with the plasma membrane for each quantum discharged, and that these vesicles are released independently of one another.

Whether the exocytotic event captured by freeze-fracture is an immediately reversible phenomenon (Pfenninger and Rovainen, 1974) or proceeds to total incorporation of the vesicle membrane into the presynaptic membrane (Heuser and Reese, 1973) is still a matter of controversy (for review see Boyne, 1978). There are many proponents of the view that vesicle membrane is totally incorporated into the presynaptic membrane (Heuser and Reese, 1973; Pysh and Wiley, 1974; Model, Highstein and Bennett, 1975; Schaeffer and Raviola, 1975; Lynch, 1982). Evidence for their position has come from attempts to deplete terminals of their vesicles by inducing a high rate of secretion with sustained electrical stimulation or depolarizing solutions. In most of the early work, dramatic changes in the number of vesicles were not noted (Hubbard and Kwanbunbumpen, 1968; Jones and Kwanbunbumpen, 1970a, 1970b). More recent attempts to deplete synapses of their vesicle population using rapid (greater than 10

Hz) stimulation or high potassium solutions have shown depletion or a significant reduction in vesicle numbers (Ceccarelli, Hurlbut and Mauro, 1972, 1973; Heuser and Reese, 1973; Pysh and Wiley, 1974; Boyne, Bohan and Williams, 1975; Schaeffer and Raviola, 1975; Lynch, 1982).

One factor common to all of these studies is that they relied upon measurements in chemically fixed tissues and it is known that chemical fixatives themselves produce a limited amount of neurotransmitter release (Smith and Reese, 1980). While not negating the results, fixation makes it difficult to know to what extent the results were representative of true cellular events.

Further evidence of total incorporation of vesicular membrane has come from work with isolated nerve endings. Subcellular fractionation techniques have permitted isolation of pinched-off nerve terminal preparations (Hebb and Whittaker, 1958). These "synaptosomal" preparations (Whittaker, Michaelson and Kirkland, 1964) retain the functional integrity of intact nerve terminals. They respond both to potassium depolarization (Blaustein, 1975) and electrical stimulation (Zimmerman and Whittaker, 1974). From work in synaptosomes, the vesicular and presynaptic membranes have been shown to be similar in their lipid composition, except for ganglioside content (Morgan, Zanetta, Breckenridge, Vincendon and

Gombos, 1973). Incorporation of the vesicular membrane into that of the terminal would thus result in a change in the composition of the terminal membrane. According to the fluid mosaic model of membrane structure (Singer and Nicholson, 1972), proteins and glycoproteins would be likely to diffuse from the patches of incorporated vesicular membrane to mix with the components of the plasma membrane. It has been shown immunocytochemically that during exocytosis, synaptic vesicle-specific components are incorporated into the plasmalemma (Jones, Walker, Stadler and Whittaker, 1982b; see also Heuser and Reese, 1981).

B. Membrane recycling

If synaptic vesicles release their contents by fusing with the presynaptic membrane, the origin of replacement synaptic vesicles must be explained. In a study of crayfish NMJs, Bittner and Kennedy (1970) calculated that if a synaptic vesicle is equivalent to a quantum of transmitter and the synaptic vesicles fuse with the presynaptic membrane, then the loss of synaptic vesicle membrane due to fusion would amount to almost 24

mm^2 of membrane/hour. Their conclusion was that a loss of this magnitude could not be sustained by the nerve terminal unless vesicle membrane were recycled from the presynaptic membrane.

Assuming exocytotic release of neurotransmitter, a local recycling mechanism was proposed to account for membrane economy and geometric constancy of presynaptic nerve terminals (Ceccarelli, Hurlbut and Mauro, 1972). Using frog cutaneous pectoris nerve-muscle preparations, Ceccarelli, Hurlbut and Mauro (1972) found evidence for such recycling. They found that approximately 80% of the transmitter is released from the nerve terminals within about 4 hours (with 2 Hz stimulation), although the terminals looked normal and contained many synaptic vesicles. (After 6-8 hours, however, the population of vesicles, like the store of transmitter, was almost completely depleted.) Many vesicles in the stimulated preparations were filled with horseradish peroxidase (HRP) from the bathing medium while control preparations showed no uptake of this extracellular tracer. Based on their findings, these investigators concluded that during stimulation the vesicles fuse with the plasmalemma, release their transmitter and are returned directly to the axoplasm. The depletion of synaptic vesicles seen with 6-8 hours of stimulation was attributed to a terminal stage in the secretion process, due to the collapse of the

mechanisms responsible for membrane recycling. In a further study (Ceccarelli, Hurlbut and Mauro, 1973) these same investigators re-emphasized that vesicles can re-form both during and after stimulation and that the reformed vesicles can store and release transmitter.

Further confirmation of the vesicle recycling theory was provided by Heuser and Reese (1973, 1975) using frog sartorius muscle preparations. Using higher rates of stimulation (10 Hz for 15 minutes), they found that after 15 minutes, 60% of the synaptic vesicles had disappeared from the nerve terminal. Concomitant with the synaptic vesicle depletion, they noted an increase in the amount of presynaptic membrane and the appearance of numerous cisternal structures, shown previously by Korneliusssen (1972a). Calculations by Heuser and Reese (1973) indicated that the loss of synaptic vesicle membrane was equal to the amount of membrane incorporated into the cisternae plus the amount by which the terminal plasma membrane increased. Based on experiments using HRP as an extracellular marker, they postulated that the following sequence of events takes place in a nerve terminal upon stimulation. Synaptic vesicles release their transmitter by coalescing with the terminal plasma membrane at the active zones. The synaptic vesicle membrane then becomes an integral part of the presynaptic membrane. Equal amounts of membrane are recovered from the terminal plasma

membrane by the formation of coated vesicles. The coated vesicles fuse to form cisternae and in the process, lose their coats. New (that is, recycled) synaptic vesicles bud off from the newly formed cisternae.

A number of studies, all of chemically fixed tissue, point to the existence of some form of local membrane recycling in a wide variety of synapses (Holtzman, Freeman and Kashner, 1971; Dickinson and Reese, 1973; Heuser and Reese, 1973; Pysh and Wiley, 1974; Zimmerman and Whittaker, 1974; Model, Highstein and Bennett, 1975; Schaeffer and Raviola, 1975; Theodosis, Dreifuss, Harris and Orci, 1976; Fried and Blaustein, 1976, 1978; Zimmerman and Denston, 1977a; Gennaro, Nastuk and Rutherford, 1978; Wagner and Kelly, 1979; Lentz and Chester, 1982; Janka and Jones, 1982; Jones, Walker, Stadler and Whittaker, 1982a, 1982b; Lynch, 1982; Morris and Nordman, 1982; Dickinson-Nelson and Reese, 1983). At present, most effort is directed toward determining the nature of the mechanism and the intermediate membrane forms involved.

1. Synaptic vesicles recycled directly from plasma membrane

Some authors feel that synaptic vesicles reform directly from the plasma membrane, without any membranous intermediates (Ceccarelli, Hurlbut and Mauro, 1972, 1973; Trubatch and VanHarreveld, 1981). Freeze-fracture studies on chemically fixed CNS tissue (Streit, Akert, Sandri, Livingstone, and Moor, 1972; Pfenninger and Rovainen, 1974) revealed dimples which the authors regarded as permanent attachment sites for synaptic vesicles. The synaptic vesicles were thought to attach to these sites upon depolarization of the bouton, open and release their transmitter, and pinch back off directly into the cytoplasm.

Boyne, Bohan and Williams (1975) proposed a slightly different mechanism. They found a decreased synaptic vesicle population with stimulation in the Narcine electric organ, a completely cholinergic tissue. They noted no significant increase in any membranous intermediates but did observe an increase in membrane interdigitations between terminals. They felt that, upon release, synaptic vesicles fused with the presynaptic membrane to cause these interdigitations but then were recycled directly from the plasma membrane.

2. Recycling intermediates

a. Cisternae

Many investigators regard cisternae as intermediate structures formed during the vesicle recycling process, either directly from the plasmalemma or by fusion of coated endocytic vesicles (eg. Heuser and Reese, 1973; Model, Highstein and Bennett, 1975; Schaeffer and Raviola, 1975; Lynch, 1982). This position is based on observations that cisternal numbers increase with synaptic activity.

In at least some cases, cisternae apparently form directly by invagination and pinching off of plasmalemma. Kadota and Kadota (1982) recently reported that, in cat superior cervical ganglion, retrieval of uncoated membrane ("macropinocytosis") occurs within 1-5 seconds following transmitter release, while coated vesicle endocytosis begins several seconds later.

b. Coated vesicles

Many investigators feel that coated vesicles play some role in retrieval, either by internalizing membrane or by sorting out synaptic vesicle-specific proteins. These organelles were first observed by Gray (1961) in neurons and extensively described by Roth and Porter (1964). Roth and Porter showed them to be involved in the selective uptake of yolk protein in mosquito oocytes. They suggested that coated vesicles lost their coats, fused, and became larger droplets within the cell, storing material that had been introduced into the cell by micropinocytosis.

Since then coated vesicles have been detected in many cell types (Friend and Farquhar, 1967; Griffiths, Warren, Stuhlfauth and Jockusch, 1981; Salisbury, Condeelis, Maihle and Satir, 1982; Brown, Anderson and Goldstein, 1983; Pilch, Shia, Benson and Fine, 1983) including neurons (Gray, 1961). It is thought that coated vesicles arise from coated pits, concave invaginations of the plasma membrane with clathrin coating on their cytoplasmic surface. Their involvement in receptor-mediated endocytosis has been firmly established, although their exact role is still a matter of controversy.

Kanaseki and Kadota (1969) further characterized

coated vesicles and proposed a mechanism by which the bristly coat on these vesicles might produce membrane infolding and endocytosis. Clathrin, the highly ordered, structural protein of the coat has been extensively characterized (Pearse, 1976; Crowther and Pearse, 1981; Garner and Lasek, 1981; Pearse and Crowther, 1981; Unanue, Ungewickell and Branton, 1981; Pearse, 1982). It has been localized immunocytochemically in nerve endings in various neural tissues (Cheng, Byrd, Whitaker and Wood, 1980; Cheng and Wood, 1982; Bloom and Puszkin, 1983), and its transport down the axon has been studied (Garner and Lasek, 1981).

Morphological evidence of coated vesicle involvement in synaptic membrane recycling has been provided in a variety of systems. In neurosecretory terminals of the posterior pituitary, Douglas and coworkers saw an increase in the number of coated vesicles with stimulation and labeling of them with HRP from the extracellular space (Douglas, Nagasawa and Schulz, 1971; Nagasawa, Douglas and Schulz, 1971.) Heuser and Reese (1973) also observed an increase in the number of coated vesicles with stimulation in frog NMJs. These coated vesicles were also labeled with HRP, indicating that at one time they were open to the extracellular space. Gennaro, Nastuk and Rutherford (1978) postulated that at the NMJ, synaptic vesicles reform directly from the coated

vesicles. They found no intermediate structures in frog NMJs; large vesicular structures produced upon stimulation proved, in their preparations, to be axolemmal infoldings containing Schwann cell processes.

It has been proposed that coated vesicles may provide a mechanism whereby synaptic vesicle-specific proteins could be re-segregated and internalized (Brown, Anderson and Goldstein, 1983). Antibodies to synaptic vesicle-specific membrane components have been used to demonstrate that synaptic vesicle components are incorporated into the plasma membrane (vonWedel, Carlson and Kelly, 1981). Coated vesicles (and coated pits) may be involved in selective re-internalization or re-segregation of the synaptic vesicle components from the plasma membrane.

IV. Non-vesicular transmitter release

One of the neurochemical discoveries that has been inconsistent with the vesicle recycling hypothesis is that newly synthesized ACH is preferentially released (Large and Rang, 1978a, 1978b). This would mean that recycled vesicles must be quickly and preferentially reloaded with transmitter and moved back into position close to the active zone. This constraint is not in keeping with evidence that recycled vesicles (loaded with extracellular

tracers) are scattered randomly through the vesicle population (Holtzman, Freeman and Kashner, 1971; Ceccarelli, Hurlbut and Mauro, 1973; Teichberg, Holtzman, Crain and Peterson, 1975).

Because choline acetyltransferase, the enzyme responsible for the synthesis of ACH, is found in nerve terminal cytoplasm, some investigators have proposed a "cytosol hypothesis" of ACH release (as opposed to the "vesicle hypothesis") to account for this preferential release of newly synthesized ACH (Tauc, 1979; Dunant, 1981). According to their hypothesis, the most recently synthesized ACH constitutes a cytosolic pool that is preferentially released. Quantization would be accomplished by a transiently activated gated channel in the presynaptic membrane and the synaptic vesicles would serve as a reservoir to replenish cytoplasmic ACH as it becomes depleted.

The proponents of this theory do not deny the evidence that vesicles undergo exocytosis or that vesicle membrane may be recycled, but contend that this process serves a function other than transmitter release. One suggested function is the extrusion of excess intracellular calcium (Dunant, 1981).

Parducz, Kiss and Joo (1976) provided another possible explanation for diminished vesicle numbers with stimulation. The vesicular membrane contains

phosphatidylcholine, a potential precursor for choline. They found evidence to support the theory that, in cases of choline deficiency, the nerve terminal is forced to use membrane-bound choline to maintain transmitter synthesis, possibly depleting vesicle numbers.

V. Turnover of synaptic membrane

Underlying the rapid, local and sporadic cycling of synaptic vesicle membrane that occurs during and immediately after stimulation, is a mechanism for replenishing the pool of synaptic vesicle membrane. The most likely source of this new synaptic vesicle membrane is the SER (Korneliussen, 1972a; Droz, Rambourg and Keonig, 1975), an organelle that is transported down the axon by fast axoplasmic transport (Tsukita and Ishikawa, 1980).

Retrograde transport of old synaptic vesicle membrane is also possible, presumably in degradative organelles or their precursors, multivesicular bodies (MVBs) (Grafstein and Forman, 1980; LaVail, Rapisardi and Sugino, 1980; Tsukita and Ishikawa, 1980). Studies in cultured fetal rat spinal cord (Teichberg, Holtzman, Crain and Peterson, 1975) showed increased numbers of labeled MVBs (containing HRP from the extracellular space) with

stimulation causing the authors to postulate that synaptic vesicle membrane turnover involves retrograde axonal transport of membrane by MVBs, as well as a local retrieval system for exocytosed membrane. Because they saw an increase in MVBs (in cat superior cervical ganglion) after stimulation, Kadota and Kadota (1982) suggested that retrograde transport of membrane for degradation is increased by stimulation. Lynch (1982) showed that, in frog sartorius nerve-muscle preparations stimulated in vitro at 2 Hz for more than 24 hours, there was a conservation of total membrane in the nerve terminal. This conservation of membrane suggests that membrane exchange between axon and nerve terminal occurs at a slow rate, relatively unaffected by synaptic activity. The local mechanism for the recycling of synaptic vesicle membrane appears to be capable of maintaining membrane constancy even during prolonged activity of the nerve terminal.

Most studies investigating membrane recycling in the presynaptic nerve terminal have been done using chemically fixed tissue (Ceccarelli and Hurlbut, 1972, 1973; Heuser and Reese, 1973; Pysh and Wiley, 1974; Model, Highstein and Bennett, 1975; Lynch, 1982). The possibility of artifacts caused by the chemical fixatives complicates the interpretation of results. A preservation technique which enables recycling to be studied without

the interference of fixation artifacts is rapid-freezing. Because this process preserves tissue more quickly than chemical fixation, it also allows for a more accurate reconstruction of temporal events. Further morphological investigations of membrane recycling, conducted with rapid-frozen material, might then provide answers to questions concerning the fate of synaptic vesicle membrane, the roles of coated vesicles and cisternae, and the rate of the recycling process.

VI. Effects of calcium on the presynaptic nerve terminal

Calcium plays several roles in neurotransmission at the neuromuscular junction. The stage which is most sensitive to small changes in extracellular calcium concentration is the secretion of neurotransmitter from motor nerve terminals (Rahamimoff, 1976). A number of studies have demonstrated that calcium ions are involved in the normal release of ACH at the neuromuscular junction (DelCastillo and Stark, 1952; Katz and Miledi, 1965a, 1965b, 1965c, 1967; Rubin, 1970; Miledi, 1973; Krnjevic, 1974; Llinas and Nicholson, 1975; Ceccarelli and Hurlbut, 1980a, 1980b). Depolarization of the presynaptic nerve causes an increase in calcium conductance. This increased conductance leads to calcium influx, probably through

channels at or near the active zones. Calcium entry causes the synaptic vesicles to fuse with the presynaptic membrane and release their transmitter.

The balance between calcium entry and the calcium-removing processes determines the intracellular calcium concentration. From a resting concentration of 10^{-7} M, an increase in free intracellular calcium concentration to 10^{-6}-10^{-5} M is needed to cause transmitter release (Baker and Knight, 1980). This increase in intracellular calcium is transient. The time course of calcium buffering in synaptosomes (after a single stimulus) has been shown to be about 1-2 msec (Blaustein and Rasgado-Flores, 1981).

It is unlikely that the abrupt fall in intracellular calcium concentration after transmitter release can be accounted for simply by extrusion from the terminal by plasmalemmal calcium pumps (McGraw, Nachsen and Blaustein, 1982). Some calcium may bind passively to cytoplasmic proteins with a high affinity for calcium (eg. calmodulin) (Blaustein and Rasgado-Flores, 1981), while some may be actively sequestered in intraterminal organelles. It has been shown that mitochondria in synaptosomes have a low affinity, high capacity storage system for calcium while a non-mitochondrial membrane compartment has a higher affinity but a lower capacity (Blaustein and Rasgado-Flores, 1981).

VII. Calcium-sequestering organelles

A. Cytoplasmic proteins

Calmodulin has been found in high concentrations in synaptosomes and has been shown to modulate the effects of calcium on synaptic vesicle transmitter release and membrane interactions. Calcium binding to calmodulin initiates phosphorylation of synaptic proteins and this phosphorylation offers a molecular explanation for calcium's regulatory effect on synaptic function (Burke and DeLorenzo, 1982; DeLorenzo, 1982). The interaction of calcium and calmodulin may serve to draw the synaptic vesicles to the membrane or to trigger the fusion of opposed membranes.

Calmodulin may play an additional regulatory role by binding calcium and thus removing it from the cytoplasmic pool. This would be a function it shares with other cytoplasmic proteins that nonspecifically bind calcium. A variety of proteins with low affinity for calcium have been isolated from nerve cells. They are likely to have a low capacity and their concentration may be limited. More efficient buffering is accomplished by organelles that actively accumulate calcium.

B. Non-mitochondrial membranous compartments

1. Synaptic vesicles

Some morphological studies suggest that synaptic vesicles bind calcium, perhaps in the very process of being stimulated to exocytosis. Bohan, Boyne, Guth, Narayan and Williams (1973) found dense particles in synaptic vesicles isolated from electric organs and fixed in high calcium solutions. Politoff, Rose and Pappas (1974) have shown that when frog NMJs are fixed in the presence of high concentrations of calcium, dense particles appear in the synaptic vesicles and mitochondria, as well as in muscle mitochondria and SER. Since such particles are not present in tissue fixed in calcium-free solutions (containing EGTA), it is presumed that they are calcium deposits. In stimulated preparations fixed in high calcium, the electron dense deposits in the synaptic vesicles disappeared, while those in the mitochondria and in the muscle SER remained. With 1-2 hours rest before chemical fixation, calcium deposits returned to control locations. The authors suggested that the decrease in synaptic activity following intense stimulation might somehow result from transient deactivation of a calcium binding capacity of the synaptic vesicle membrane. Boyne, Bohan and Williams (1975) also

saw a decrease in the number of synaptic vesicles with calcium binding capacity after stimulation of Torpedo electric organ. They interpreted this as an indication that the ability to bind calcium is essential for fusion with the presynaptic membrane and subsequent transmitter release.

There is also limited evidence that synaptic vesicles actually sequester calcium in an energy-dependent manner. Rephaeli and Parsons (1982) have shown that calmodulin stimulates ATP-dependent uptake of calcium into Torpedo synaptic vesicles, probably acting through a phosphorylation mechanism.

The results of the studies summarized in the preceeding paragraph must be examined with caution as the studies were all conducted in chemically fixed tissues under non-physiological conditions.

In the only studies using rapid-freezing, rather than chemical fixation, to approach the problem of calcium localization calcium has been detected by x-ray probe microanalysis in the synaptic vesicles of frog nerve terminals (Becker, Canada and Pappas, 1982) and Torpedo electric organs (Phillips and Boyne, 1982).

2. SER/Cisternae

The SER has also been proposed as a principal calcium-sequestering organelle in nerve endings (Henkart, 1975; Blaustein, Ratzleff, Kendrick and Schweitzer, 1978; Blaustein, Ratzleff and Schweitzer, 1978; Somlyo, Somlyo, Schuman, Scarpa, Endo and Inesi, 1981). Morphological definitions of the SER vary greatly with investigator. Early papers simply viewed SER as a broad subcategory of membrane organelles while current investigators are more likely to describe specifically the organelle they define as SER. In rapid-frozen, freeze-substituted squid axons, both SER (unspecified form), and mitochondria were shown by x-ray microprobe analysis to accumulate calcium with calcium loading and oxalate injection (Henkart, Reese and Brinley, 1978). In a study of chemically fixed tissue, Lynch (1982) observed a reduction of SER (narrow, angular, branched membranous profiles) in frog NMJs after stimulation, raising the possibility that it swells to contribute to the population of cisternal structures seen in these synapses.

Calcium has also been localized by x-ray microprobe analysis in SER (flattened, branching cisterns; structure determined by serial section reconstruction) and large vesicular profiles in CNS synaptosomes (McGraw, Somlyo and Blaustein, 1980). When oxalate was included in

the fixative calcium-oxalate deposits were present in intraterminal mitochondria, SER cisterns and large vesicular profiles.

C. Mitochondria

The ability of mitochondria to sequester calcium has been well established (Lehninger, 1970; Alnaes and Rahamimoff, 1975; Rahamimoff, 1976; Lehninger, Reynafarje, Vercesi and Tew, 1978; Rahamimoff, Erulkar, Lev-Tov and Meiri, 1978; Nicholls and Akerman, 1981). A number of investigators have shown that isolated mitochondria swell as they sequester calcium (Greenawalt, Rossi and Lehninger, 1964; Peachy, 1964; Hackenbrock and Caplan, 1969) and mitochondria that have accumulated large amounts of calcium contain electron-dense granules (presumably calcium phosphate) in electron micrographs (Greenawalt, Rossi and Lehninger, 1964; Peachy, 1964; Lehninger, Reynafarje, Vercesi and Tew, 1978).

Mitochondrial swelling correlates with nerve stimulation in chemically fixed synapses (Heuser and Reese, 1973; Pysh and Wiley, 1974; Lynch, 1982; but see Parducz and Joo, 1976). It has been suggested that the swelling may be at least partially an artifact of chemical fixation, possibly due to increasing permeability of mitochondrial membranes to phosphate (Hackenbrock and

Caplan, 1969), increasing the permeability of the plasmalemma to calcium, or to the presence of calcium as a contaminant in glutaraldehyde (Oschman and Wall, 1972).

Rapid-freezing offers a solution to this problem. To date, only two studies investigating mitochondrial swelling and calcium sequestration using rapid-freezing have been carried out. Although they did not measure mitochondrial dimensions, Ornberg and Reese (1980) showed that mitochondria in rapidly stimulated, rapid-frozen terminals, freeze-substituted with oxalate, exhibit a dense calcium-containing deposit in their matrix. Hirokawa, Heuser and Evans (1981) demonstrated that mitochondria swell and acquire granules in rapid-frozen, oxalate-substituted terminals electrically stimulated in high (10 mM) calcium Ringer's. They did not examine terminals that had been allowed to undergo a period of recovery or terminals stimulated in normal (1.8 mM Ca) Ringer's.

Calcium sequestration in the small and complex presynaptic terminal is difficult to study. Many of the organelles present in the terminal are potentially involved in the process and the reactions of presynaptic organelles to chemical fixation (Birks, 1971; Clark, 1976; Smith and Reese, 1980) greatly complicate matters. Further morphological investigation of the calcium sequestration question should be conducted in rapid-frozen

material where chemical fixation artifacts are minimized.

VIII. Presynaptic cytoskeleton

Like calcium sequestering organelles, cytoskeletal elements in the presynaptic nerve terminal, including microtubules and neurofilaments, may also be affected both by calcium entry during neurotransmission and by chemical fixation. The slow en masse movement of these organelles down the axon in the slow component of anterograde axonal transport may be responsible for axonal growth (Peters and Vaughn, 1967). Microtubules have also been implicated as a substrate for the rapid transport of membrane-bound organelles (for review see Grafstein and Forman, 1980). During nerve growth, axonal microtubules are continually added to at the cell body (Heidemann, Landers and Hamborg, 1981). To regulate their extension at the nerve terminal, disassembly promoted directly by calcium, and through calmodulin-mediated mechanisms, has been proposed (Lasek and Black, 1977; Schlaepfer, Zimmerman and Micko, 1981).

Since microtubules are calcium-sensitive they are difficult to study in chemically fixed tissue. Calcium is a component of most fixatives and is difficult to eliminate completely. It has also been shown that

fixatives themselves (glutaraldehyde) can alter the morphology of microtubules by changing them to linear beaded structures (Himes, Jordan and Wilson, 1982).

IX. Dense-cored vesicles

Another organelle observed in chemically fixed nerve terminals to have changed with stimulation is the dense-cored vesicle (DCV). DCVs exist as major or minor components in most synapses and have been shown to contain any of a number of peptides and amines. These substances may function as neurotransmitters (as in aminergic synapses; for review see Cooper, Bloom and Roth, 1982) or as modulators of synaptic activity (ChanPalay, Engel, Wu and Palay, 1982). Lynch (1980) showed a decrease in the number of DCVs in frog NMJs with either 2 Hz stimulation for 24 hours, or 30 Hz stimulation for 0.3 hours. This decrease in number was supported in a study by Philippe and Tremblay (1981) in chick ciliary ganglion. They saw a similar decrease with 100 Hz stimulation for 15 minutes. (Paradoxically, however, they saw an increase in DCV numbers with 10 Hz stimulation for 15 minutes (Philippe and Tremblay, 1983).)

X. Tissue preservation: chemical fixation or rapid-freezing?

Most of the morphological studies that have investigated presynaptic organelles and their roles in, and reaction to, neurotransmission have used chemically-fixed tissues. The earliest electron microscopists used either osmium tetroxide or potassium permanganate as primary fixatives (Palay, 1954; Luft, 1956). Later studies used various aldehydes, introduced in 1963 by Sabatini, Bensch and Barrnett, as primary fixatives, followed by secondary fixation with osmium tetroxide. Since then, glutaraldehyde has found increasing favor as the aldehyde of choice.

In defining the process of fixation, Baker (1960) suggested that tissue fixation should prevent autolysis, bacterial attack and changes in volume or shape. Hayat (1970) added that the aims of fixation were rapid preservation of structure with a minimum alteration from the living state, as well as protection from deformation during the processing and sectioning of the tissue. On the ultrastructural level, chemical fixation approaches these goals but introduces problems of its own.

Fixation using aldehydes takes seconds to minutes to penetrate living tissue and organelle and membrane

rearrangements are possible during this time. Neurofilaments and microtubules, for example, can undergo depolymerization (Hayat, 1981), or otherwise react to fixative (Himes, Jordan and Wilson, 1982). At the NMJ, significant numbers of vesicles may undergo exocytosis (Smith and Reese, 1980). The osmolarity and ionic content of the fixative also have been shown to be significant factors in the production of artifacts. Vesicles can shrink, or clump together, and membranes can be disrupted (Birks, 1971; Clark, 1976). Lee, McKenzie, Kobayashi, Garfield, Forrest and Daniel (1982) showed that glutaraldehyde exerted osmotic pressure on tissues in that a higher concentration of glutaraldehyde or an extended fixation time caused cell shrinkage. They also found evidence that tissues are still osmotically reactive after glutaraldehyde fixation, implying further possible changes during buffer washes and post-fixation procedures.

Aldehyde fixatives alone have been shown to have a stimulatory effect on synapses. Increases in MEPPs of up to 50/sec were seen during fixation of frog cutaneous pectoris nerve-muscle preparations (Smith and Reese, 1980). An increased number of synaptic vesicle openings were seen in freeze-fractured preparations. Failure to block the observed increase in transmitter release with reduced calcium concentrations or increased magnesium, the authors felt that this increased quantal release was

calcium-independent. Hypertonic solutions have also been shown to cause calcium-independent increases in quantal transmitter release (Kita and VanderKloot, 1977). Since 3% glutaraldehyde (the concentration of the fixative used by Smith and Reese) is 300 mM, in addition to the Ringers in which it is dissolved, it is possible that the changes seen could be related to hyperosmotic effects. However, Smith and Reese showed a persistence of the fixative effects, even when the aldehyde Ringer's was mixed to an isosmotic level. This suggested that the aldehyde effect shared properties with, but was fundamentally different from, the osmotic effect.

Chemical fixation can also produce artifactual membrane structures. For example, flattened or ellipsoid vesicles, once thought to be a reliable indication of an inhibitory synapse (Uchizono, 1965) may be an artifact of chemical fixation (Valdivia, 1971; Korneliussen, 1972b). In fact, Nakajima and Reese (1983) have demonstrated that inhibitory terminals innervating crayfish stretch receptors have round synaptic vesicles when they are preserved by rapid freezing, whereas previous studies in aldehyde fixed material showed them to contain flattened synaptic vesicles.

In a freeze-fracture and thin-section study of developing fibroblasts, Hasty and Hay (1978) found that large, vesicle-containing blisters of membrane were an

artifact of glutaraldehyde fixation. Mounds or "blebs" (similar to the blisters in fibroblasts) were identified in growth cones of cultured neurons and thought to be the location of new membrane addition. Work by Rees and Reese (1981), using rapid-freezing and freeze-substitution, showed that these mounds were artifacts found only when tissue was processed with aldehyde fixatives.

Findings such as these raise doubts about the validity of conclusions regarding membranous recycling intermediates when those conclusions are based on fixed tissue and are frequently contradictory, even in a single system such as the frog NMJ.

Rapid-freezing is becoming an accepted alternative to aldehyde fixation. Rapid-freezing consists of freezing fresh unfixed tissue so rapidly that ice crystals do not form. It is being used routinely by investigators in several different tissues (VanHarreveld and Trubatch, 1975; Heuser, Reese, Dennis, Jan, Jan and Evans, 1979; Trubatch and VanHarreveld, 1981; Dudek, Childs and Boyne, 1982; Pysh, Florek and Burlew, 1982; Schnapp and Reese, 1982).

Early attempts at directly freezing tissue involved immersing it in cryogenic liquids (Fernandez-Moran, 1960; Inoue, Kurosumi and Deng, 1982). A technique introduced later was touching the tissue against a highly conductive metal surface cooled by liquid

nitrogen or liquid helium (VanHarreveld and Crowell, 1964; Heuser, Reese and Landis, 1974; Boyne, 1979; Escaig, 1982; Pysh, Florek and Burlew, 1982). With rapid-freezing, membrane rearrangements are avoided and ion and water movements minimized (Ornberg and Reese, 1980). After freezing, cellular structure can be exposed by freeze-fracture or permanently stabilized by subsequent freeze-substitution (Feder and Sidman, 1958). The latter process dehydrates the solidly frozen tissue with an organic solvent, usually acetone, and simultaneously fixes it with osmium or another fixative soluble in the organic medium. The tissue can then be brought to room temperature for embedding (Feder and Sidman, 1958; Ornberg and Reese, 1981; Harvey, 1982; Schnapp and Reese, 1982).

A limitation of rapid-freezing is that only the most superficial 20 μm of tissue on the surface which first contacts the metal surface is frozen rapidly enough (in 1-2 msec) to avoid distortion by ice crystals (Heuser, Reese, Dennis, Jan, Jan and Evans, 1979). This limitation necessitates careful selection of an experimental model in which the area of interest is contained within this tissue depth. For this reason the cutaneous pectoris muscle of the frog (which has a thickness of 80 μm) has traditionally been chosen for studies of NMJs using rapid-freezing.

Artifacts due to compression might be expected

with rapid-freezing. Although the tissue hits the cooled metal surface with high velocity, it freezes so rapidly that no obvious distortion of the fine structure occurs (VanHarreveld and Steiner, 1970; Pysh, Florek and Burlew, 1982). Quantitative evidence for lack of compression was provided by Bernard and Krigman (1974). They compared the ratio of the long and short axes in sections of neural processes and found no difference between rapid frozen and glutaraldehye fixed material. Further evidence was provided by VanHarreveld and Trubatch (1979) by measuring long and short axes of gelatin slices before and after freezing. The area was found to change significantly only in slices 200 μm and larger.

Many studies executed with chemically fixed material deserve re-evaluation with rapid-frozen tissue. Synaptic vesicle fusion and depletion, mitochondrial changes and alteration of cytoskeletal structure are just a few of the morphological phenomena which might be attributed at least partially to interaction of the tissue with chemical fixatives.

It was the purpose of this study to use rapid-freezing to determine the morphological changes produced in frog nerve terminals by tetanic stimulation. Experiments were conducted on the frog cutaneous pectoris

nerve-muscle preparation and material was preserved by either rapid-freezing or chemical fixation. Stimulation-induced changes in tissues preserved by these two very different protocols were compared and attempts made to correlate the morphological changes observed in rapid-frozen tissue with the physiological processes involved.

The cutaneous pectoris muscle was chosen for these experiments for several reasons. It has been used by a number of other investigators for both physiological and anatomical studies (Dreyer and Peper, 1974; Gennaro, Nastuk and Rutherford, 1978; Ceccarelli and Hurlbut, 1980a, 1980b; Magleby and Miller, 1981; Johnston, Kravitz, Meiri and Rahamimoff, 1983), so information derived from the present study could be compared to previous ones. It is conveniently dissected and produces an isolated preparation with little or no trauma to the muscle or its nerve. Of special importance to these experiments is the fact that the muscle is only about 80 μm thick. Since rapid-freezing penetrates about a 20 μm depth of tissue without artifact, about 1/4 of the muscle and its nerve terminals are well-preserved.

Among the new observations made in this study were two that warranted additional experiments. The first was that synaptic vesicle diameter increased after stimulation and decreased with subsequent rest. It was postulated

that this might be a result of ACH loading, calcium sequestration or osmotic forces.

The second observation involved the response of mitochondria to stimulation. It was hypothesized that this mitochondrial shape change was due to calcium sequestration.

A series of six experimental groups used rapid-freezing to investigate the effects of different divalent cation concentrations in the extracellular bathing medium. One pair of experimental groups consisted of stimulated and unstimulated preparations soaked in normal Ringer's solution. A second pair consisted of stimulated and unstimulated preparations maintained in 10 mM Ca Ringer's solution (rather than in the normal, 1.8 mM Ca Ringer's). The final pair consisted of stimulated and unstimulated preparations maintained in 10 mM Mg Ringer's, with no added calcium. Since stimulation causes an increase in intracellular calcium concentration and this increased intracellular calcium may mediate the morphological effects of stimulation, the effects of stimulation should be exaggerated by stimulation in a Ringer's solution where the calcium concentration is 10 mM instead of 1.8 mM. Because the action of magnesium is antagonistic to calcium at nerve terminals, no effects of stimulation should be noted in the terminals stimulated in a Ringer's solution in which calcium has been replaced by

magnesium.

Another series of three experimental groups investigated the effects of hemicholinium-3 (HC-3) on nerve terminals. One group was soaked in 100 uM hemicholinium Ringer's solution, one was soaked in hemicholinium Ringer's and then stimulated, while the third was soaked, stimulated and rested, all in hemicholinium Ringer's solution. HC-3 has been shown to interfere with the synthesis of ACH by blocking the high-affinity uptake of choline into nerve terminals (Birks and MacIntosh, 1957). Previous investigators, using HC-3 in stimulation experiments followed exclusively by chemical fixation, have produced conflicting results. Jones and Kwanbunbumpen (1970a) and Ceccarelli and Hurlbut (1975) observed a decrease in the number of synaptic vesicles after stimulation in HC-3 while Heuser and Reese (1973) observed no further reduction in synaptic vesicle number with stimulation in HC-3 after stimulation in normal Ringer's solution. Others have hypothesized that the nerve terminal, when ACH synthesis is blocked by HC-3, can overcome this interference by breaking down synaptic vesicle membrane to yield phosphatidylcholine, a possible choline precursor (Parducz, Kiss and Joo, 1976). If this latter suggestion is true, a severe depletion of synaptic vesicles would be expected after stimulation in HC-3. In addition, if the increase in synaptic vesicle volume seen

after stimulation in the first set of experiments was related to ACH loading, the presence of HC-3 in the bathing medium during stimulation should block the volume increase.

MATERIALS AND METHODS

I. Animals

Fifty-three cutaneous pectoris nerve-muscle preparations were dissected from pithed juvenile (four weeks post-metamorphosis) Rana pipiens. Thirty of these preparations were used in the first set of experiments and were obtained from the University of Michigan Amphibian Facility in Ann Arbor, Michigan. A later series of experiments was conducted using juvenile Rana pipiens supplied by Hazen Farms, Vermont. The 23 cutaneous pectoris nerve-muscle preparations from these animals were dissected as in the first study.

II. Experimental groups

A. Experimental preparations

Experimental preparations in the first set of experiments were divided into four groups (each containing five muscles), two that were stimulated and two that were stimulated and rested (see Table I). Stimulated nerve-muscle preparations were indirectly electrically stimulated (stimulus delivered to the nerve rather than

TABLE I

EXPERIMENTAL GROUPS

A. First Set of Experiments

	Musc	Micr	Ringer's	Stimulation	Primary Fix	Second Fix	Blockstain	Dehyd
Chemically fixed control	5	15	1.8 mM Ca	---------	3% glutar	OsO_4 ferr	------	MetOH
Chemically fixed stimulated	5	15	1.8 mM Ca	15 Hz;15 min	3% glutar	OsO_4 ferr	------	MetOH
Chemically fixed rested	5	15	1.8 mM Ca	15 Hz;15 min	3% glutar	OsO_4 ferr	------	MetOH
Rapid-frozen control	5	15	1.8 mM Ca	---------	freezing	OsO_4	10% UA	acetone
Rapid-frozen stimulated	5	15	1.8 mM Ca	15 Hz;15 min	freezing	OsO_4	10% UA	acetone
Rapid-frozen rested	5	15	1.8 mM Ca	15 Hz;15 min	freezing	OsO_4	10% UA	acetone

B. Second Set of Experiments

Normal control	2	15	1.8 mM Ca	---------	freezing	OsO_4	10% UA	acetone
Normal stimulated	2	15	1.8 mM Ca	15 Hz;15 min	freezing	OsO_4	10% UA	acetone
Calcium control	2	15	10 mM Ca	---------	freezing	OsO_4	10% UA	acetone
Calcium stimulated	2	15	10 mM Ca	15 Hz;15 min	freezing	OsO_4	10% UA	acetone
Magnesium control	2	15	10 mM Mg	---------	freezing	OsO_4	10% UA	acetone
Magnesium stimulated	2	15	10 mM Mg	15 Hz;15 min	freezing	OsO_4	10% UA	acetone
Hemicholinium control	3	15	100 um HC	---------	freezing	OsO_4	10% UA	acetone
Hemicholinium stimulated	3	15	100 um HC	15 Hz;15 min	freezing	OsO_4	10% UA	acetone
Hemicholinium rested	3	15	100 um HC	15 Hz;15 min	freezing	OsO_4	10% UA	acetone

directly to the muscle) with a suction electrode. Stimulation was performed at room temperature (23-25°C) in a Ringer's bath for 15 minutes at 15 Hz. Stimuli were delivered as square wave pulses 0.2 msec in duration and at a strength 3-4 times that required to produce a twitch of maximal strength (judged by microscopic inspection). One minute after the cessation of the stimulus these preparations were either rapid-frozen or chemically fixed by immersion. Rested nerve-muscle preparations were stimulated as above but allowed to rest for 60 minutes before being frozen or fixed.

A second set of experiments contained five experimental groups (see Table I). A series investigating the effects of different divalent cation concentrations had three experimental preparations. In these experiments two nerve-muscle preparations were soaked for 15 minutes in either normal (1.8 mM Ca), high calcium (10 mM Ca), or high magnesium (10 mM Mg) Ringer's solution and then stimulated for 15 minutes at 15 Hz (at room temperature). These muscles were then rapid-frozen within one minute of cessation of the electrical stimulus.

To investigate the effects of hemicholinium-3 (HC-3), a blocker of ACH uptake into the nerve terminal, another series of experiments was conducted which contained two experimental preparations, three muscles in each. One experimental group was soaked in 100 um HC-3

Ringer's for 45 minutes, followed by 15 minutes of 15 Hz stimulation before rapid-freezing. The other experimental group was also soaked for 45 minutes and stimulated for 15 minutes but was rested in HC-3 Ringer's for 60 minutes prior to rapid-freezing.

B. Control preparations

All of the above experimental groups contained a number of corresponding control preparations. Control nerve-muscle preparations of the first set were unstimulated and either rapid-frozen or chemically fixed.

In all preparations in the first set of experiments a 330 Kohm resistor was installed to control for possible resistance built up at the electrode tip and subsequent reduction in current flow to the nerve. In four preparations fresh Ringer's solution was cycled at 5 ml/minute to control for proper oxygenation of the preparation. Since no difference in muscle contraction was observed between experiments with the freshly cycled Ringer's, this technical modification was omitted from the other experiments in the first set. Neither of these modifications was included in the second set of experiments.

As a control to measure for artifacts due to stretch (possibly induced by pinning the preparation to

Sylgard), the periodicity of cross-striations in longitudinal sections of muscles from the six groups of the first set of experiments were measured at the light microscopic level. No differences were found between any of the groups; all averaged measurements of 3 μm from Z-band to Z-band. Had stimulation or physical manipulation of the preparations induced stretching of the muscle (and hence of the NMJ) an increase in size of the regions between the A-bands (the I-bands) would have been observed. Therefore, it is safe to assume that any stretch artifacts that may have been introduced were consistent in all preparations.

Control preparations (two muscles in each of the three control groups) in the calcium/magnesium experiments were soaked in their respective Ringer's solutions for 30 minutes at room temperature before preservation by rapid-freezing. Hemicholinium controls (three muscles) were soaked in 100 μM HC-3 Ringer's solution for 60 minutes and immediately frozen.

Curare blocks neurotransmission at the NMJ by acting as a competitive antagonist to ACH at its receptor on the muscle membrane. As a control, two muscles from the second set of frogs were soaked at room temperature for 5 minutes in curarized Ringers (.75 μg/ml), then indirectly stimulated for 15 minutes at 15 Hz. This curare concentration was determined by testing for the

minimal dosage that blocked muscle contraction. Preparations were frozen one minute following cessation of electrical stimulus. During analysis of the data, these controls were compared to preparations stimulated in normal Ringer's solution to ensure that changes seen after stimulation were due to presynaptic, rather than postsynaptic, events. By quantitative measurement these curare muscles were morphologically identical to those stimulated in normal Ringer's.

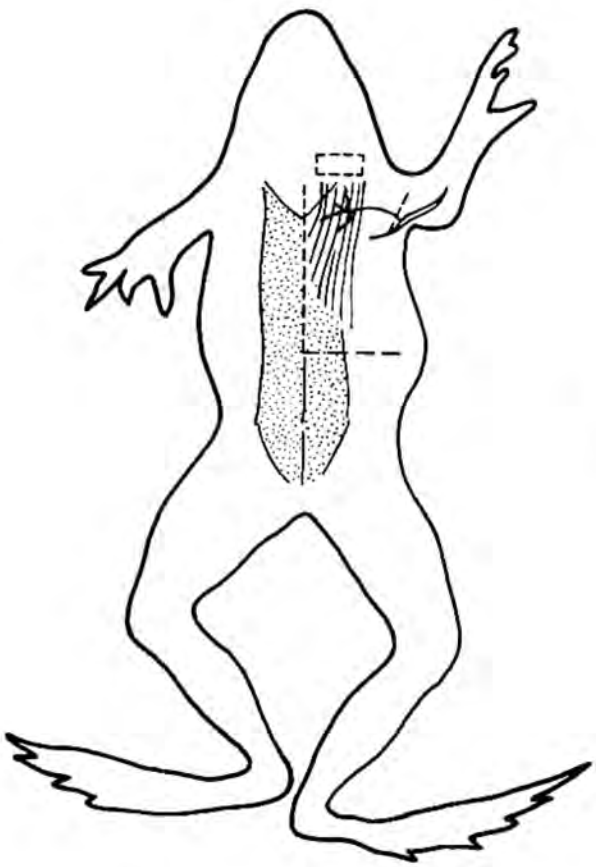
Osmolarity of all Ringer's solutions was controlled by reducing the amount of NaCl to allow for increased magnesium or calcium. Osmolarity measured by an Advanced Instruments osmometer was 200-250 mM for all solutions. All solutions had a pH of 7.00-7.20.

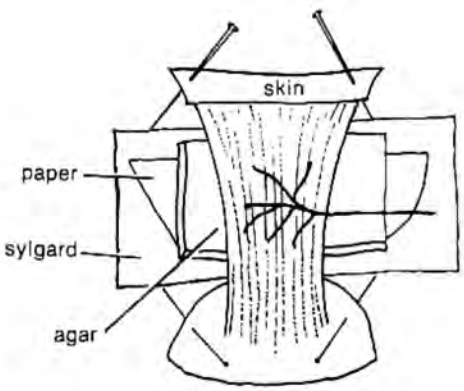
III. Dissection

The frogs were pithed and pinned out, ventral surface up, in a Sylgard (silicone polymer, Dow Corning Co., Midland, MI.) dish filled with room-temperature Ringer's solution. Using a dissecting microscope, the abdominal skin was pulled up and trimmed off along a line connecting the forelegs. The cut was made inferior to the origin of the cutaneous pectoris muscle (Figure 1A). A second parallel cut was made in the throat skin superior to the origin to produce a thin band of skin containing

Figure 1. Diagrams of cutaneous pectoris nerve-muscle preparation.

A diagram of a Rana pipiens showing the cutaneous pectoris muscle and the course of the nerve which innervates it (A) shows the nerve leaving from a main trunk in the axilla and crossing the cutaneous pectoris muscle perpendicular to the longitudinal axis of the muscle fibers. During dissection the nerve to the cutaneous pectoris was severed at its branch point from the axillary trunk. The cutaneous pectoris muscle was teased away from its midline attachment to the sternum. Cuts were made rostral to the muscles's origin in throat skin (saving a rectangular piece of skin), and caudal to its insertion in the connective tissue of the anterior abdominal wall. The nerve-muscle preparation was then removed from the animal and pinned out. A diagram of the dissected nerve-muscle preparation (B) shows how the rostral end of the muscle was left attached to a flap of throat skin while the caudal end was attached to abdominal musculature. These attachments provided a convenient means of pinning the muscle to a rectangle of Sylgard without touching the muscle itself. The 3-5 mm nerve trunk was never touched with forceps and was easily aspirated into a suction electrode for stimulation of the muscle.





A B

the origin of the cutaneous pectoris muscle.

The large lateral thoracic muscle was cut away to expose the nerves and vessels of the axilla. The nerve to cutaneous pectoris runs from the axilla to the lateral border of the muscle. The nerve was freed from surrounding connective tissue and snipped as far proximal as possible, leaving a 3-5 mm trunk. The distal part of the nerve was never handled.

The skin band was cut in the midline to separate the origins of the paired muscles. The skin band was pulled back gently to expose the cutaneous pectoris muscle origin in the middle third of the skin band. Using a minuten pin probe, the cutaneous pectoris muscle was bluntly dissected from the underlying chest muscle and from its connective tissue attachments along the midline, to its insertion at the xiphoid process of the sternum and the anterior abdominal musculature.

A low cut was made, between the tendinous intersections of the rectus abdominus muscle, across the entire anterior abdominal wall. Cuts were made up the sides of the abdomen and across the thoracic wall above the freed cutaneous pectoris muscle, as close to the muscle as possible. The underlying peritoneal connections were removed.

The dissected preparation was then pinned out in a Sylgard dish filled with fresh room temperature Ringer's

solution (Figure 1B). Pins were placed in the throat skin flaps and the abdominal musculature. An aluminum freezing disc was anchored into the Sylgard dish and prepared for mounting the muscle preparation as follows: A Sylgard rectangle of uniform size was glued to the aluminum freezing disc. To improve the visibility of the nerve, a half-circle of white filter paper was glued to the Sylgard rectangle. To cushion the muscle and minimize the impact of hitting the freezing block, a rectangle of agar was placed on top of the filter paper. The mounting procedure consisted of stretching the muscle over these items and pinning it to the Sylgard rectangle at approximately 120% of its resting length (Ralston and Libet, 1953; Turkanis, 1973). All muscles, regardless of the type of preservation used, were mounted in the same fashion to control for the effects of stretching and contraction of the preparation during stimulation. These preparations were then refrigerated in normal frog Ringer's solution until approximately thirty minutes before use (no longer than four hours).

IV. Tissue preparation

A. Chemical fixation

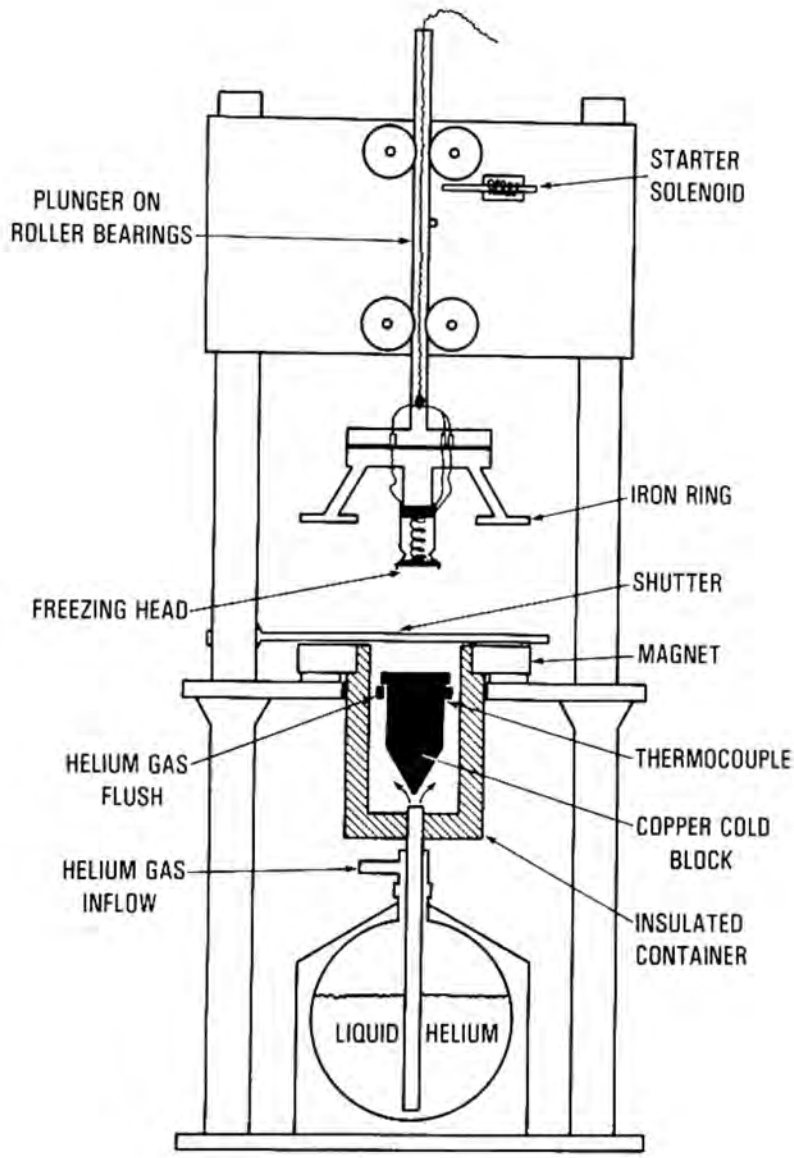
For chemical fixation, muscles were immersed in a Ringer's solution containing 3% glutaraldehyde for one hour at room temperature, washed in 90 mM cacodylate buffer (pH 7.2-7.3) at room temperature, and post fixed in 1% OsO /1.5% potassium ferrocyanide on ice for two hours (Karnovsky, 1971). Following final rinses in 90 mM cacodylate buffer, tissues were dehydrated in a series of graded methanol, passed briefly through propylene oxide and embedded in Araldite resin.

B. Rapid-freezing

Freezing was accomplished with the "Slammer" (Figure 2), where each muscle (mounted on agar and Sylgard rectangles on an aluminum disc) (Figure 3) was frozen against a polished copper block cooled to approximately -265°C (8°K) with forced liquid helium. The Slammer, an apparatus designed by Heuser, Reese and Landis (1974) for rapid-freezing biological specimens, consisted of a mechanical main frame placed on a table over a liquid helium tank (Figure 2). Held in the base of the framework was a polished copper freezing block in a cold block

Figure 2. A diagram of the Slammer.

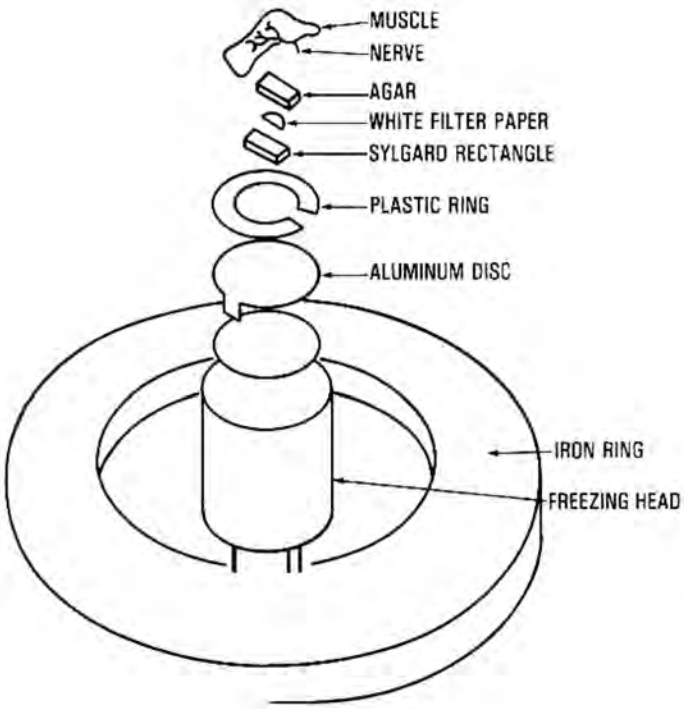
The nerve-muscle preparation was mounted on an aluminum disk prior to its experimental treatment. For freezing, the disk was quickly fastened to a detachable freezing head. The freezing head was then attached to the end of a long specimen rod. When released, the specimen rod fell and the tissue struck the polished surface of a copper block cooled to 8°K by liquid helium forced up from a tank below. The copper surface was kept free from condensation of atmospheric gases by a shutter, which snapped out of the way as the specimen rod dropped. The superficial tissue froze immediately (within 1-2 msec) upon impact with the copper block. The freezing head was detached from the specimen rod and quickly transferred to a storage container of liquid nitrogen. (Diagram modified from Heuser, Reese, Dennis, Jan, Jan and Evans, 1979)


STARTER
SOLENOID
PLUNGER ON
ROLLER BEARINGS
IRON RING
SHUTTER
FREEZING HEAD
MAGNET
HELIUM GAS
FLUSH
THERMOCOUPLE
COPPER COLD
BLOCK
HELIUM GAS
INFLOW
INSULATED
CONTAINER
LIQUID HELIUM

(2)

Figure 3. A diagram of a nerve-muscle preparation mounted for freezing.

This schematic representation demonstrates the sequential steps used in mounting the nerve-muscle preparation on the freezing head. The muscle was placed on a piece of agar to cushion its impact with the copper block and to raise its surface above the plastic ring. The agar rested on a piece of filter paper glued to a Sylgard rectangle. The filter paper provided contrast for viewing the small nerve. The Sylgard acted as a support to which the muscle was pinned to insure a constant stretch to 120% of its resting length. The Sylgard rectangle was glued to an aluminum disc which attached to the freezing head. (Diagram modified from Heuser, Reese, Dennis, Jan, Jan and Evans, 1979)


MUSCLE
NERVE
AGAR
WHITE FILTER PAPER
SYLGARD RECTANGLE
PLASTIC RING
ALUMINUM DISC
IRON RING
FREEZING HEAD

3

assembly. Liquid helium was pumped from the tank through a stainless steel transfer tube to the cold block assembly, through suitable openings in the table and base plate. Pumping the liquid helium was accomplished by pressurizing the tank with helium gas. A pressure relief valve was incorporated in the line, set between 5-7 oz/in^2. When liquid helium was not being pumped, the pressure line was vented to atmospheric pressure and the dewar top closed. A shutter above the cold block assembly deflected the helium plume to prevent prefreezing of specimens and condensation on the copper freezing block. The specimen was inverted and mounted on the underside of a rod suspended above the copper block. Triggering the release control caused the shutter to swing out of the way and the guide rod and specimen to drop onto the freezing block. The freezing process was complete in milliseconds to a tissue depth of about 20 μm.

The preparation, still mounted on its stage, was immediately transferred to liquid nitrogen, where it was freeze-substituted in 4% OsO_4/acetone. Six milliliters of 4% OsO_4 in acetone in plastic scintillation vials was frozen with liquid nitrogen. Working under liquid nitrogen in a styrofoam container, tissues rapid-frozen wtih the Slammer were transferred to the vials, one specimen per vial. The vials containing the muscles on the frozen OsO_4-acetone were filled with liquid nitrogen

and the cap was loosely screwed on.

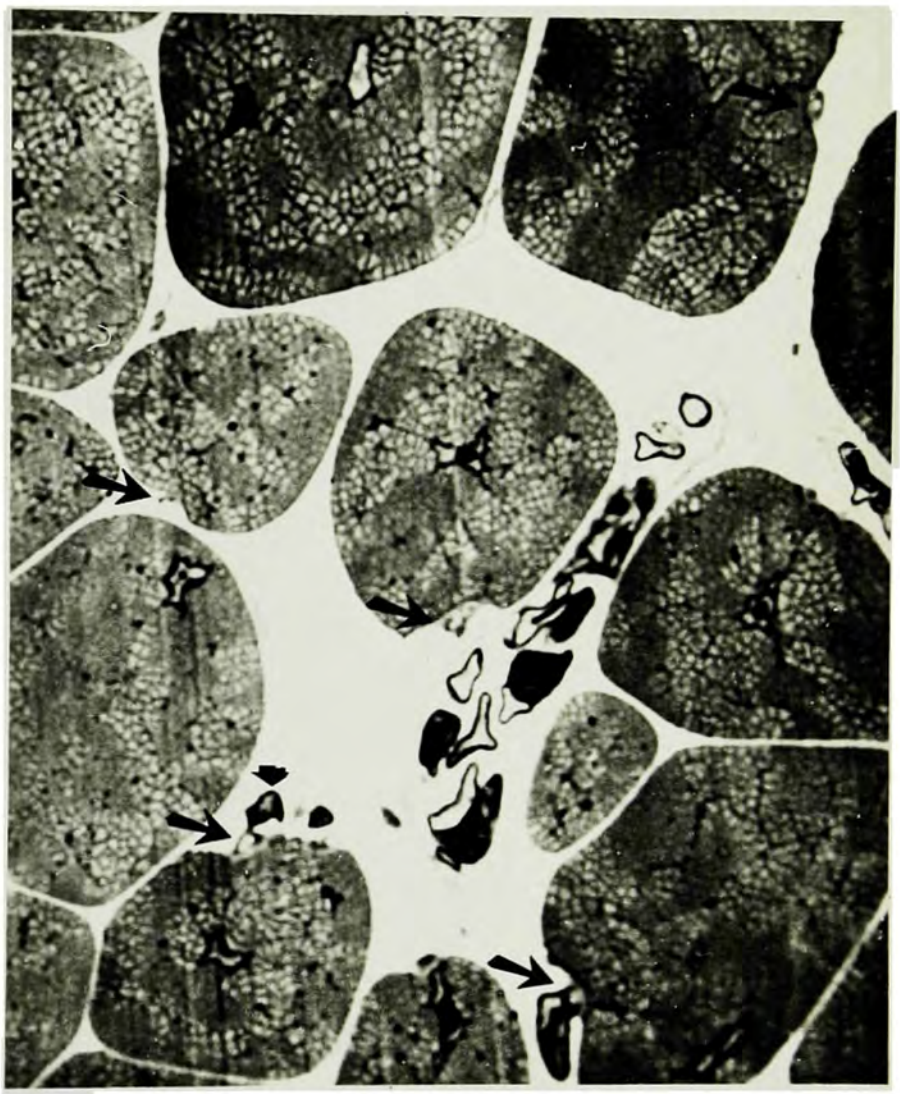
The vials were placed in a rack in a double-walled insulated container which was then filled with liquid nitrogen and placed on a shaker overnight. During this period, as the liquid nitrogen in the container and vials slowly evaporated, the temperature gradually rose, melting the OsO_4-acetone at -90°C. The still frozen tissue was then penetrated by both the OsO_4 and the acetone, becoming both fixed and dehydrated before reaching temperatures at which damaging ice crystals would otherwise form. Following acetone and methanol rinses the tissue was then block stained with 10% uranyl acetate in methanol overnight at 4°C, dehydrated and embedded in Araldite resin.

C. Electron microscopy

Thick (1 μm) sections of the embedded tissue were stained with toluidine blue to localize areas of abundant nerve terminals (Figure 4), then trimmed and thin-sectioned on an AO-Reichert Ultracut ultramicrotome with a Dupont diamond knife. Silver-grey (45nm) sections were collected on formvar- and carbon-coated slot grids. Sections were stained with 10% uranyl acetate in methanol (30 min) and .03% aqueous lead citrate (1.5 min) and examined in a Philips 400 transmission electron

Figure 4. A light micrograph of cutaneous pectoris muscle cross-section with nerve terminals.

This 1 μm section of a chemically fixed unstimulated muscle from a juvenile Rana pipiens was stained with toluidine blue and shows darkly stained muscle fibers. Nerve terminals appear as scattered, lightly staining, hat-shaped structures (arrows) on the surfaces of the muscle fibers. Some of these nerve terminals are capped by Schwann cells (arrowhead). When areas rich in nerve terminal profiles were located, the tissue block was trimmed and 45 nm (silver-gray) thin sections were taken. (magnification = 2,000X)



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microscope. Cross-sectioned nerve terminals were photographed without selection. Terminals were rejected only if they appeared grossly distorted by artifact or if the plane of section appeared to be extremely oblique, according to the following criteria: 1) The majority of mitochondria were elongated (maximum diameter greater than 3 times the minimum diameter); 2) Neurofilaments and microtubular profiles were linear; 3) The presynaptic membrane showed at least 3 active zones with Schwann cell processes interposed. Fifteen nerve terminal cross-sections were photographed for each of the muscles. Prints were enlarged 2.5x to a final magnification of approximately 75,000x. Illustrations used in this thesis were printed at either 70,500x or 63,000x.

VI. Morphometry

Direct counts were made of all of the organelles observed in each photographed nerve terminal. Measurements were made of the organelles and membranes using a Hewlett-Packard 9874A digitizer and 9835A desktop computer.

Synaptic vesicles were defined as round, electron-lucent, membrane-bounded structures. Coated vesicles were similar to synaptic vesicles but were coated with dark, spiky material on their cytoplasmic surface.

Similarly, coated plasmalemmal invagination were counted as coated pits. Flat synaptic vesicles were flattened structures, found in the same vicinity as round synaptic vesicles and having short diameters less that 1/3 their long diameters. Dense-cored vesicles were membrane bounded vesicles with a dark core.

Mitochondria were also counted and measured. They were only occasionally difficult to identify due to morphological changes undergone as a result of experimental manipulation. Those counted had a distinguishable double membrane and evidence of cristae.

Microtubules were structures 25 nm in diameter and were counted but not measured. Neurofilaments (less than 10 nm diameter) were not counted individually but their domains were outlined and measured.

Cisternae of two types were counted individually. Round cisternae were round or occasionally irregular forms while flat cisternae were broad, flattened membrane profiles. The lumina of both forms were electron-lucent and did not contain organelles or cytoplasmic matrix. Anglular tubules with very narrow lumina were counted as smooth endoplasmic reticulum.

Other complex membrane structures were regarded as prelysosomal organelles or residual bodies. A multivesicular body was defined as a single membrane enclosing space containing two or more vesicles. An

autophagic vacuole was a similar structure, but consisted of a double membrane enclosing a space containing amorphous material or vesicles. Multilamellar bodies consisted of tightly packed membrane, while organelles of a uniform dark texture and irregular shape were counted as dense bodies. Whorled collections of plasmalemma also were counted. Schwann cell processes were identified by the presence of ribosomes and cytoplasm denser than that of the nerve terminals. Organelles in these processes were not measured.

The circumference and diameter of all vesicular profiles were measured on enough micrographs to accumulate 300-500 measurements for each muscle. These values were then plotted as a histogram to determine a cutoff point between synaptic vesicles and cisternae (see Figure 9). Following this cutoff determination, mean synaptic vesicle diameter was computed for all profiles below the cutoff. Cisternae (profiles above the cutoff) and all other organelles were counted and measured, and the remaining synaptic vesicles counted, but not measured.

VI. Statistics

Means and variances were computed for each variable measured (see Appendix for list of variables). The variances in each experimental group were then examined for homogeneity by Bartlett's Chi-square test. Means within each group were tested for interactions using an F statistic (for groups with homogeneous variances), or an approximate F statistic (for groups with dissimilar variances) (Cochran, 1954). The simplest method of combining the measurements from the different muscles in any given experimental group would have been to take the arithmetic mean of the measurements. If, however, the experiments appeared to be of different precision (heterogeneous variance), some kind of weighted mean was more precise. Cochran (1954) provided recommendations about the kinds of weighted means and standard errors that were appropriate and the situations in which they were preferred. The appropriately weighted mean ($\bar{x}$), variance (s^2), and degrees of freedom (df) were then determined for each experimental group. Student's T-Test was used to compare the means of pairs of experimental groups (see Appendix). Statistical significance was determined at an α-level of .01. Only those differences that were statistically significant at $p < .01$ are discussed.

RESULTS

I. First set of experiments

A. Chemically fixed nerve-muscle preparations

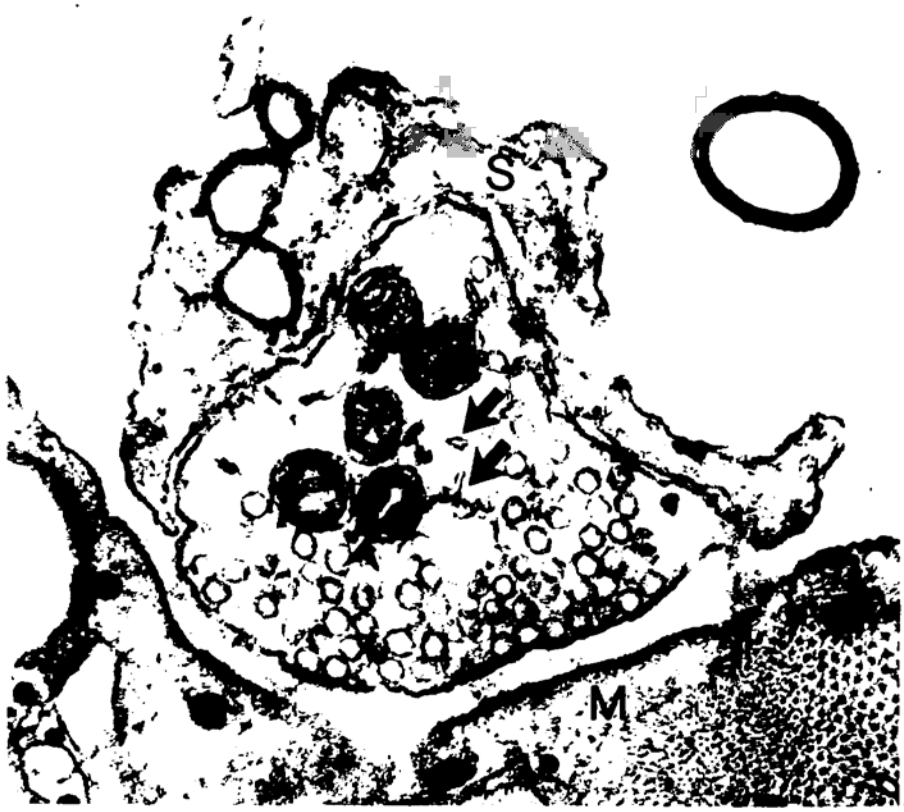
1. Control preparations

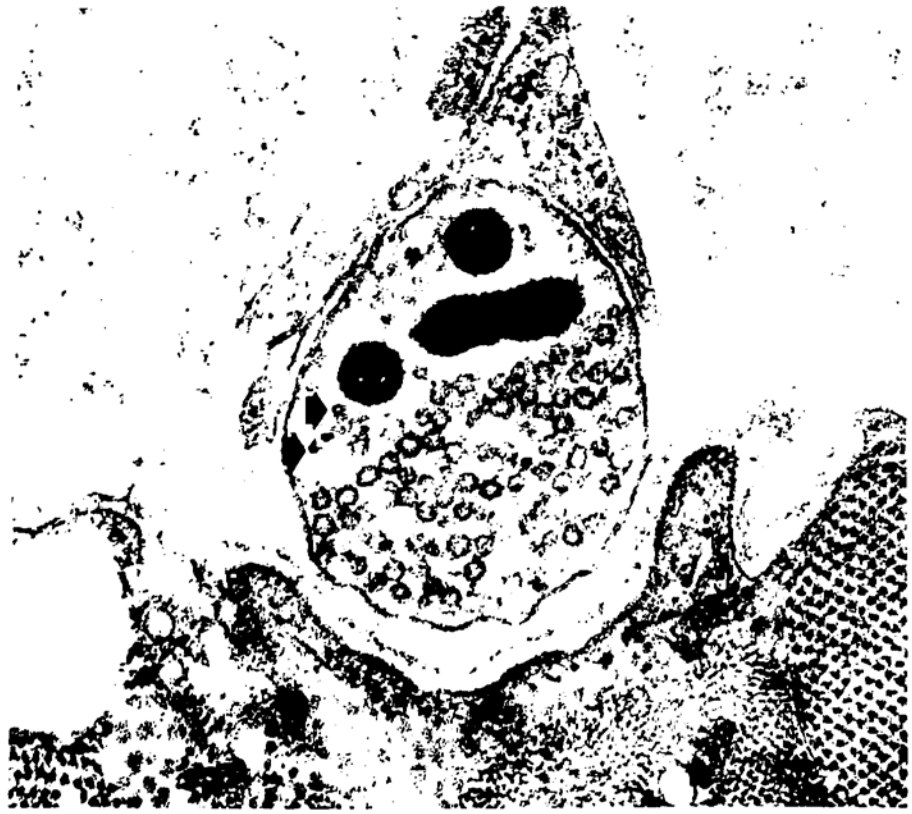
Chemically fixed nerve terminals were examined first to determine which of the stimulation-induced changes reported by previous investigators were reproducible in the present study. Cross-sections of nerve terminals from control preparations that were chemically fixed without prior stimulation demonstrated a normal complement of presynaptic organelles (Figure 5A). The nerve terminals contained numerous vesicular structures: synaptic vesicles (both round and flat), dense-cored vesicles (DCVs), occasional coated vesicles, round cisternae and larger flattened cisternae. Mitochondria, microtubules, neurofilaments, smooth endoplasmic reticulum (SER), and glycogen constituted other organelles that were consistently seen in these nerve terminals.

Figure 5. Electron micrographs of chemically fixed and rapid-frozen control nerve terminal cross-sections.

A chemically fixed control (unstimulated) nerve terminal (A) from a juvenile Rana pipiens is shown in the center of the micrograph, capped by a Schwann cell(S) and resting on a postsynaptic muscle fiber (M). The nerve terminal shows a normal complement of presynaptic organelles; eg. mitochondria (which occasionally contain electron-dense granules; double arrowhead), synaptic vesicles,and SER (arrows).

A rapid-frozen control terminal (B) shows organelles similar to the fixed control nerve terminal. The round synaptic vesicles are smaller than those in the chemically fixed terminals. Mitochondria are smaller and more electron-dense. Occasional microtubules are seen (short arrows). (magnification = 70,500X)





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2. Stimulated preparations

Fixed stimulated nerve terminals were swollen; that is, their cross-sectional area was greater (Table II). The mitochondria were greatly enlarged (increased in both diameter and volume, Figure 17B; Table II) and had disrupted cristae (Figure 6A). The number of synaptic vesicles per terminal profile decreased 30% with stimulation (Figure 12). The number of flattened synaptic vesicles, while remaining low, increased (Table II). The loss of synaptic vesicles was paralleled by increases in the plasmalemma (ie. terminal circumference) (Table II) and in the sizes of cisternal profiles (Figures 14A, 14B, and 15). A previously unreported finding was that microtubules decreased with stimulation (by 24%; see Table II).

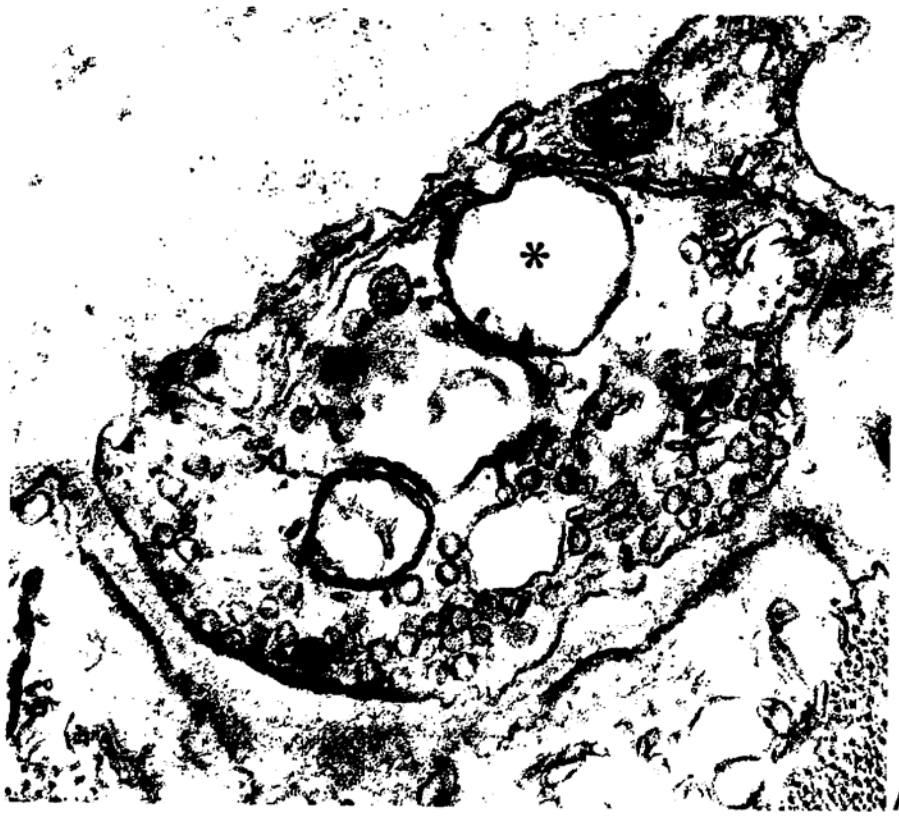
3. Rested preparations

A third experimental group was also stimulated but was allowed to rest for 60 minutes prior to immersion in the fixative (Table I). The rested presynaptic terminals remained only slightly swollen; average terminal cross-sectional area and circumference had decreased from stimulated values but the area had not completely returned to control measurements (Table II). The sizes of the

Figure 6. Electron micrographs of chemically fixed and rapid-frozen stimulated nerve terminal cross-sections.

A chemically fixed stimulated nerve terminal (A) from a juvenile Rana pipiens demonstrates greatly enlarged and disrupted mitochondria (*). The number of round synaptic vesicles decreased with stimulation while the number of flat synaptic vesicles increased (arrow). Round and flat cisternae are present.

A rapid-frozen stimulated nerve terminal (B) shows slightly enlarged mitochondria which are less electron-dense (m) than mitochondria in the frozen controls. The number of round synaptic vesicles decreased after stimulation and individual synaptic vesicles increased in size. The number of cisternal structures also increased (c). Dense-cored vesicles were occasionally seen (short arrows). (magnification = 70,500X)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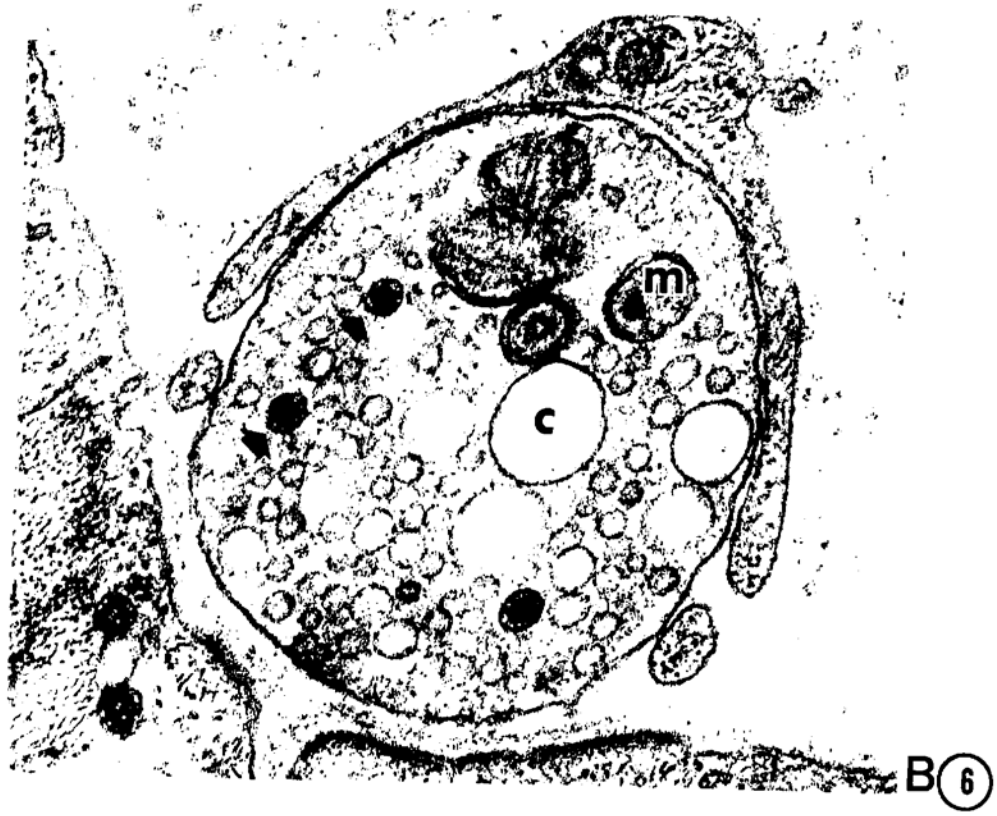
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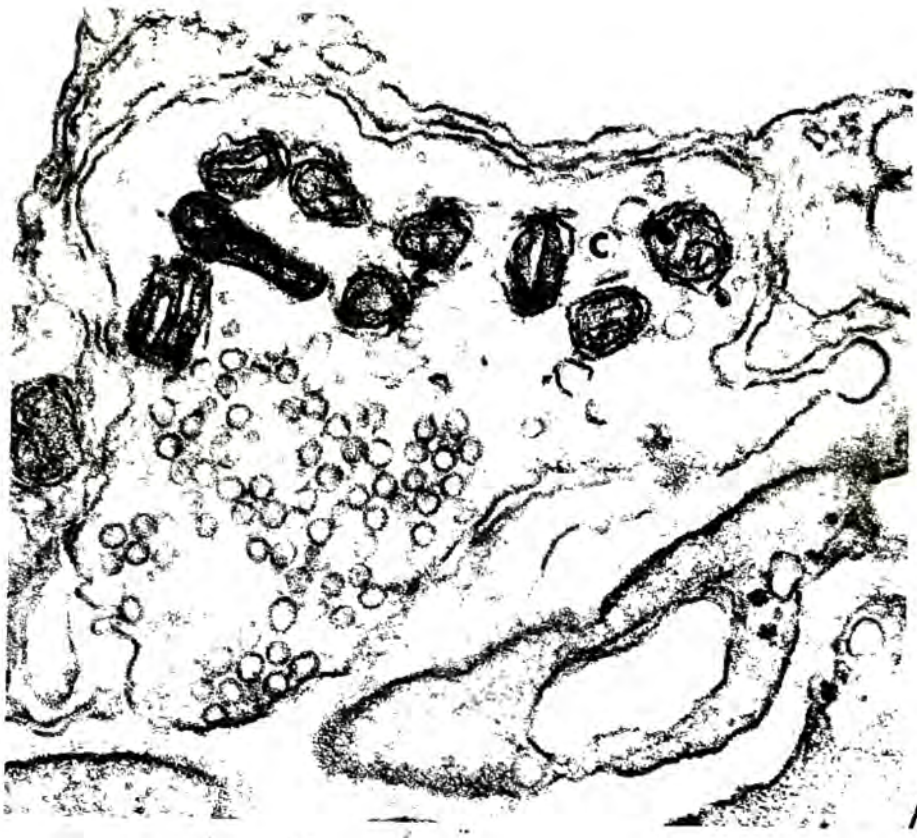
Figure 7. Electron micrographs of chemically fixed and rapid-frozen rested nerve terminal cross-sections.

A chemically fixed nerve terminal that was rested for 60 minutes after stimulation (A) has normal mitochondria. Numbers of round synaptic vesicles were near control values in such terminals. Some cisternal profiles were still present (C).

A rapid-frozen rested nerve terminal (B) demonstrated slightly enlarged mitochondria. Numbers of round synaptic vesicles returned to control values and individual vesicles regained control sizes. Only a few cisternal profiles were evident (C). (magnification = 70,500X)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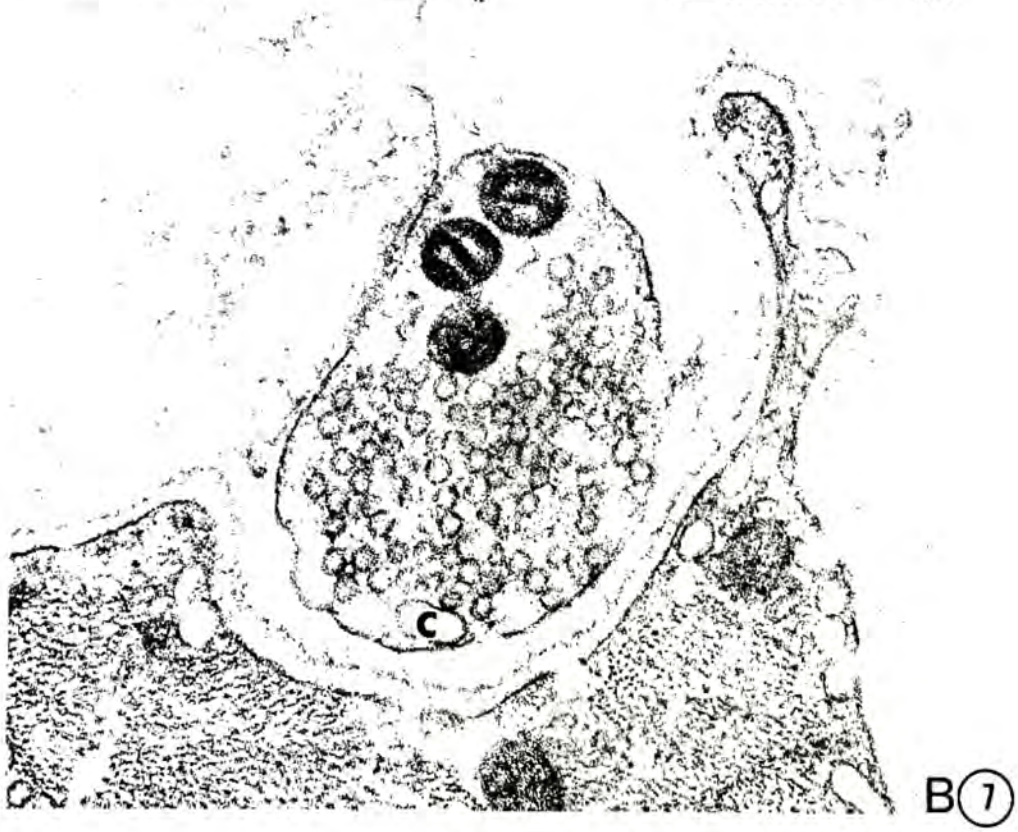
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B(1)

cisternal profiles (Figure 14) and the numbers of microtubules per terminal cross-section (Table II) had returned to normal. Mitochondria were still slightly swollen in the fixed rested nerve terminals (Figure 17C). Their number per cross-section increased over the number in the controls (Figure 16, Table II).

B. Rapid-frozen nerve-muscle preparations

1. Control preparations

The frozen control terminals (Figure 5B) showed the same organelles as the fixed controls (Figure 5A). Membranes appeared smoother in the frozen material and some organelles stained differently. Attempts were made in preliminary experiments to develop protocols that minimized these staining differences but some differences still remained. Microtubules were darker and more crisp in the frozen terminals while synaptic vesicle and cisternal membranes were faded. Glycogen was not preserved in the frozen material but the intracellular network of fibrillar material was. Neurofilaments often stained poorly and were difficult to distinguish from cytoplasmic network. Also, due again to the presence of intracellular fibrillar matrix, SER was difficult to identify.

2. Stimulated preparations

Morphological changes due to stimulation were evident in the frozen experimental preparations (Figures 6B). They paralleled the changes seen in the chemically fixed material but were less pronounced (Table II). Contrary to the results in fixed tissue the terminals were not detectably swollen nor was there a measurable increase in their circumference (Table II). Cisternae increased in number with stimulation, but not in size (Figures 6B, 14 and 15). The number of synaptic vesicles per terminal profile in the frozen material decreased 33% (Figure 12). (Flat synaptic vesicles were rarely seen in any of the frozen terminals and those counted as such may have been SER.)

The number of mitochondrial profiles per terminal cross-section significantly decreased with stimulation (Figure 16; Table II). While the average cross-sectional area of individual mitochondrial profiles increased (Figure 13E; Table II), the total mitochondrial cross-sectional area per terminal did not increase.

Rapid-freezing exposed two previously unreported effects of stimulation. Microtubules decreased by 56% (Table II). In addition, the average synaptic vesicle diameter increased from a control diameter of 38.6 nm

± .0011 (SE) to a stimulated diameter of 42 nm ± .0009 (SE) (Figures 13C, 13D; Table II) ($p < .001$).

3. Rested preparations

As in the fixed series of preparations, rested terminals in the frozen series had returned toward their normal morphology (Figure 7B). The number of synaptic vesicles per terminal profile was within the control range (Figure 12), and their average diameter had decreased to 39.9 nm ± .0009 (SE) ($p < .001$) (Table II). The number of cisternal profiles was reduced, although still higher than in the controls (Figure 15 and Table II). There was also a decrease in plasmalemma relative to controls (Table II).

With 60 minutes of rest, the diameter of individual mitochondria decreased but not enough to match controls (Figure 17F; Table II). Contrary to the indications in fixed tissue, where the number of mitochondrial profiles increased beyond control values with rest, those in the frozen rested preparations remained below control values.

The effects of stimulation on a nerve terminal, as observed in rapid-frozen material, can be summarized as follows: (1) The number of synaptic vesicles decreases while the number of cisternal structures increases. (2) The average synaptic vesicle diameter increases from a

control diameter of 38.6 nm to 42 nm with stimulation. (3) Microtubules decrease in number. (4) Mitochondrial profiles decrease in number and increase in size, but the total mitochondrial cross-sectional area per terminal did not change. With sixty minutes of rest after stimulation, most organelles regain their normal morphology. The exceptions are mitochondria and microtubules.

C. Comparison between chemically fixed and rapid-frozen terminals

1. Control groups

The only difference in the treatment of the chemically fixed control and rapid-frozen control groups was their method of preservation. Most structures were smaller in the frozen control terminals, including the terminals themselves (as evidenced by cross-sectional area and circumference), mitochondria, synaptic vesicles and DCVs (Table II). Synaptic vesicle diameter, in particular, was different in the two groups (Figures 13A and 13C). Average synaptic vesicle diameter was 38.6 nm± .0011 (SE) in the frozen control terminals and in fixed control terminals was 49 nm ± .0009 (SE) ($p < .001$). Synaptic vesicle numbers, however, were within the same

range (Figure 12; Table II).

The number of cisternal profiles, both round and flat, was higher in the fixed control terminals (Figures 14A and 14C). Coated pits and coated vesicles were rare in both control groups (Table II).

Mitochondria in the frozen control material were smaller in diameter with respect to chemically fixed control preparations and their matrix appeared more homogeneous and electron-dense (Figures 5B and 17). The number of mitochondrial profiles in the fixed control terminals was lower than in the frozen controls and the total mitochondrial volume per terminal (based on total mitochondrial cross-sectional area per terminal profile) was higher (Figure 16; Table II).

2. Stimulated groups

A comparison of the chemically fixed and rapid-frozen groups that had been stimulated revealed certain fundamental differences. Only terminals in the fixed stimulated group increased in volume and circumference compared to their respective controls (Table II). Synaptic vesicle numbers were similar in the fixed and frozen stimulated groups but synaptic vesicle diameter was again significantly different (Figures 12, 13B and 13D). The synaptic vesicles in the frozen stimulated

terminals increased to an average diameter of 42.1 nm from a frozen control diameter of 38.6 nm ($p < .001$)(Table II). The synaptic vesicles in the fixed stimulated terminals, on the other hand, were larger than in the frozen (50.6 nm $\pm$ .0008(SE) as opposed to 42.1;$p < .001$), remaining close to their control diameter of 49 nm $\pm$.0009(SE).

There were more cisternal profiles per terminal cross-section in the frozen stimulated terminals (Figure 11). Most of these were round; flat cisternae were much less prominent than in the fixed nerve terminals (Figures 14B and 14D; Table II). Examination of serial sections of both fixed and frozen terminals revealed that two round cisternae often joined to form a single flat cistern. Rarely it was observed that round cisternae were continuous with multivesicular bodies (MVBs). MVBs were counted and measured in all micrographs, but their occurrence was rare and no pattern to their presence was noted.

Coated pits and coated vesicles were present in stimulated terminals of both groups. The number of these structures was low and a statement about significance was difficult to make. However, a pattern observed was that the number of both coated pits and coated vesicles increased after stimulation and with rest returned to within control range (see Table II).

While stimulated terminals in both groups had the

same number of mitochondrial profiles per terminal cross-section (Figure 16), the individual mitochondria in the fixed stimulated preparations were increased in diameter and their combined cross-sectional area was greater (Figures 17B and 17E; Table II) than in frozen preparations. This swelling was qualitatively obvious in micrographs of fixed stimulated terminals showing swollen disrupted mitochondria (Figure 6A).

3. Rested groups

With rest, both fixed and frozen preparations showed a return to control morphology (Figures 7A and 7B; Table II). The sizes of structures seen in the frozen material remained consistently less than in the fixed. Once again, a significant difference was observed in synaptic vesicle diameters between the frozen and fixed rested terminals ($p < .001$). The frozen rested terminals contained synaptic vesicles with an average diameter of 39.9 nm ± .0009 (SE), which showed a decrease in size from stimulated values to nearly control size. The average synaptic vesicle diameter of the fixed rested terminals decreased to less than control values of 48 nm ±.0009 (SE).

Freezing revealed that after sixty minutes of rest mitochondrial profiles decreased in diameter but had not

increased back to their control numbers (Table II).

Microtubules were fewer in the frozen rested terminals, remaining at the low level seen in stimulated terminals (Table II). In the fixed terminals, in contrast, microtubules increased in number back to control counts.

II. Calcium/magnesium experiments

Experimental protocols of the normal Ringer's control and stimulated groups in this series of experiments were identical to those of the frozen control and stimulated groups, respectively, in the first set. Normal Ringer's control terminals in the calcium/magnesium series had fewer DCVs (although individually larger), fewer microtubules and less total mitochondrial cross-sectional area than controls in the first set (Table III). Terminals stimulated in normal Ringer's in the second set had fewer and larger cisternae and fewer mitochondrial profiles than in the first set. DCVs were individually larger and microtubules more numerous (Table III).

Within the calcium/magnesium series of experimental groups, control preparations soaked in normal

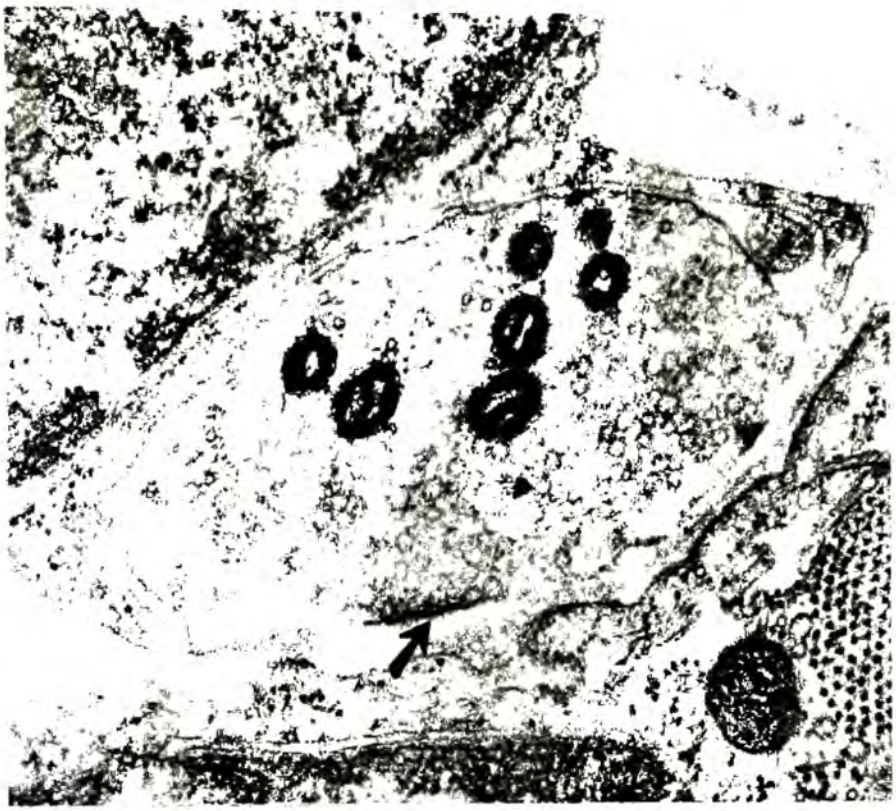
(1.8 mM Ca) Ringer's solution were compared to those soaked in 10 mM Ca Ringer's or in 10 mM Mg Ringer's. Very few differences among the three groups were noted (Figures 8A, 9A and 10A; Table III). The calcium control terminals had slightly more DCVs than normal controls. Both calcium and magnesium controls had fewer synaptic vesicles and cisternae than normal controls (Figure 18; Table III). Although the average circumference of each type of cistern increased in magnesium Ringer's solution, the reduction in cisternal numbers with magnesium resulted in less total cisternal membrane than in normal Ringer's solution (Figures 19A and 19E).

Comparison of terminals soaked in normal Ringer's solution to those stimulated at 15 Hz for 15 minutes revealed changes which generally paralleled those in the frozen stimulated terminals of the first set of experiments (Figures 8A and 8B; Tables II and III). The number of synaptic vesicles per terminal decreased (by 44%) with stimulation (Figure 18). This was coupled with increases in the size of cisternal profiles and increases in the total amount of cisternal membrane (Figures 19A, 19B and 20). No increase in the number of cisternae was detectable, however (Table III). There were indications of mitochondrial changes (Figures 21A and 21D; Table III), but the only statistically significant change was an increase in the total mitochondrial cross-sectional area

Figure 8. Electron micrographs of normal Ringer's control and stimulated nerve terminal cross-sections.

Rapid-frozen control nerve terminals from juvenile Rana pipiens in 1.8 mM Ca Ringer's (A) with a normal complement of presynaptic organelles, eg. mitochondria, round synaptic vesicles, a few round cisternae, microtubules and neurofilaments. This terminal also exhibits an active zone (arrow).

Rapid-frozen nerve terminals stimulated in 1.8 mM Ca Ringer's (B) showed expected changes due to stimulation. The number of synaptic vesicles was less and those present appeared slightly larger. Round cisternal structures increased in size (C). Coated vesicles were observed occasionally (arrow). This terminal demonstrated a double profile, seen occasionally, and counted as a single nerve terminal. (magnification = 70,500X)



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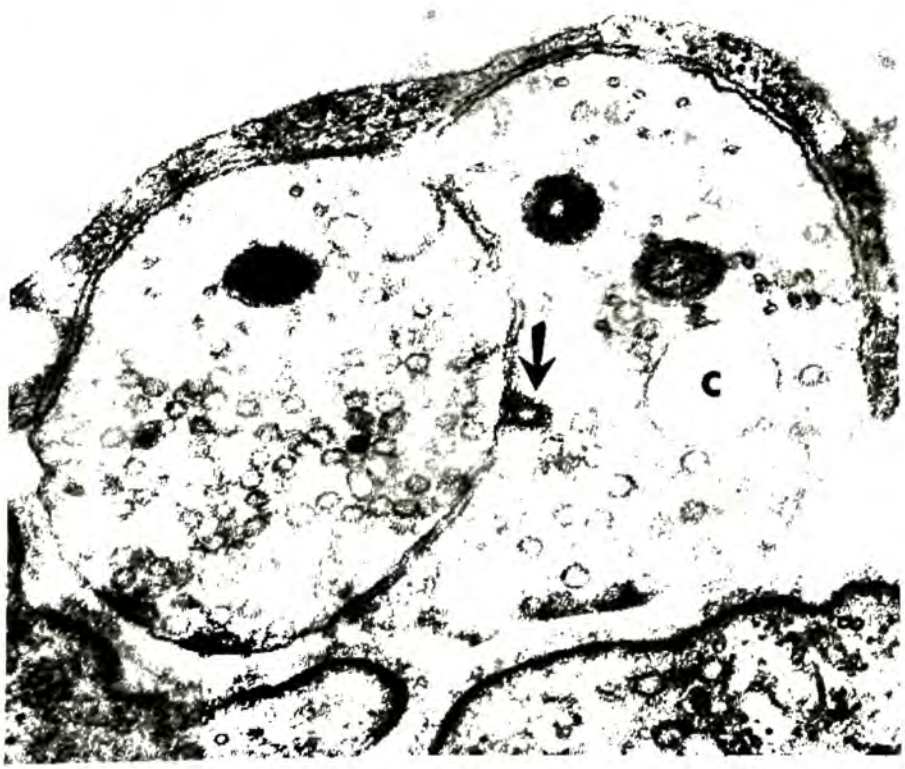

B (8)

Figure 9. Electron micrographs of calcium Ringer's control and stimulated nerve terminal cross-sections.

Rapid-frozen control nerve terminals from juvenile Rana pipiens in 10 mM Ca Ringer's solution (A) appeared morphologically identical to frozen control nerve terminals in normal (1.8 mM Ca) Ringer's solution.

Rapid-frozen nerve terminals stimulated in 10 mM Ca Ringer's solution (B) exhibited changes slightly different from those seen in terminals stimulated in normal Ringer's. As in the normal stimulated nerve terminals, mitochondrial profiles were slightly larger and less electron-dense (m) and the number of synaptic vesicles was decreased. The most striking difference was that the number of round cisternae (C) was much higher after stimulation in 10 mM Ca Ringer's. Cisternal structures observed in both the overlying Schwann cell and the postsynaptic muscle were non-specific and unrelated to cisternae in the presynaptic nerve terminal.
(magnification = 70,500X)

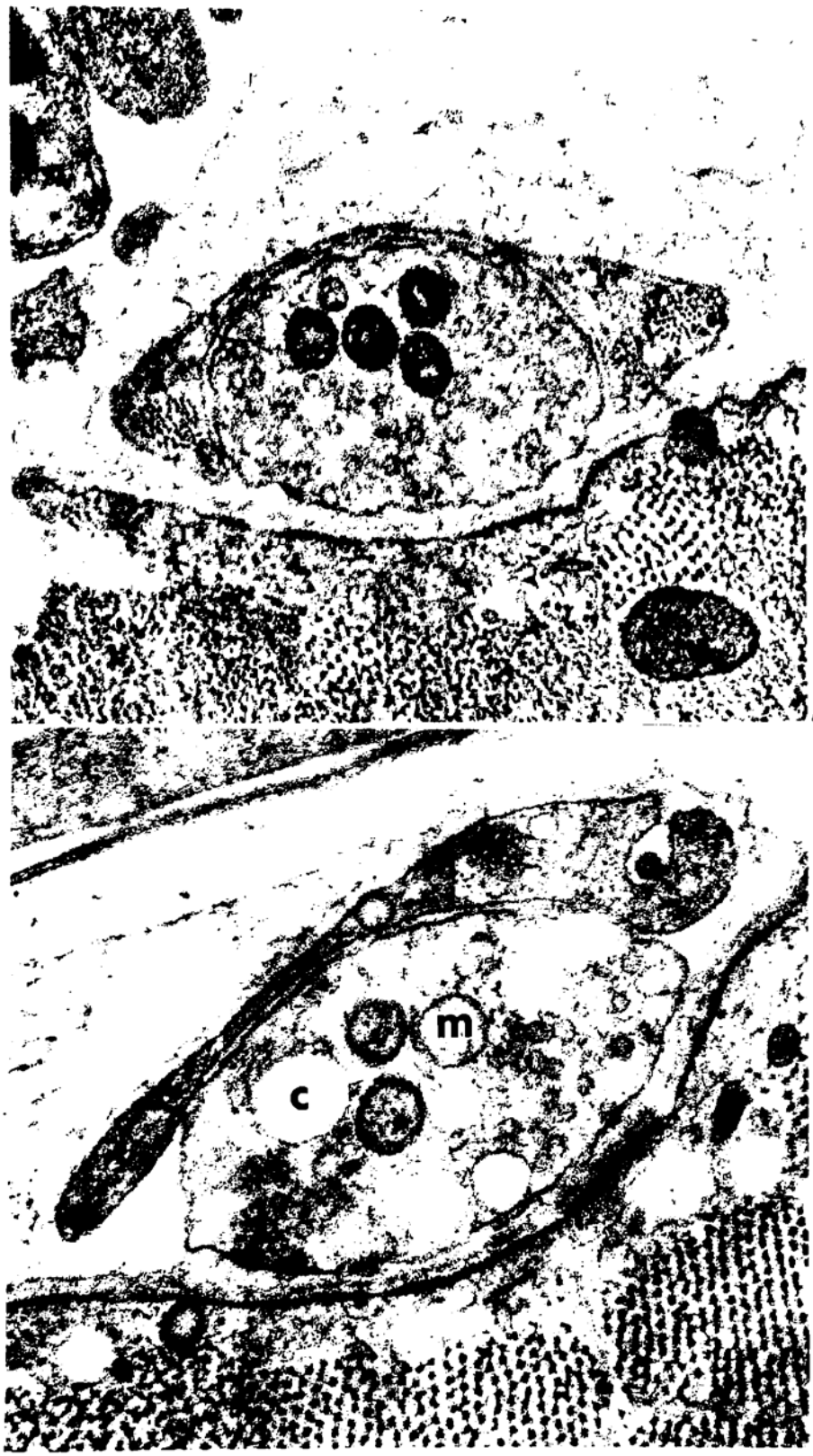

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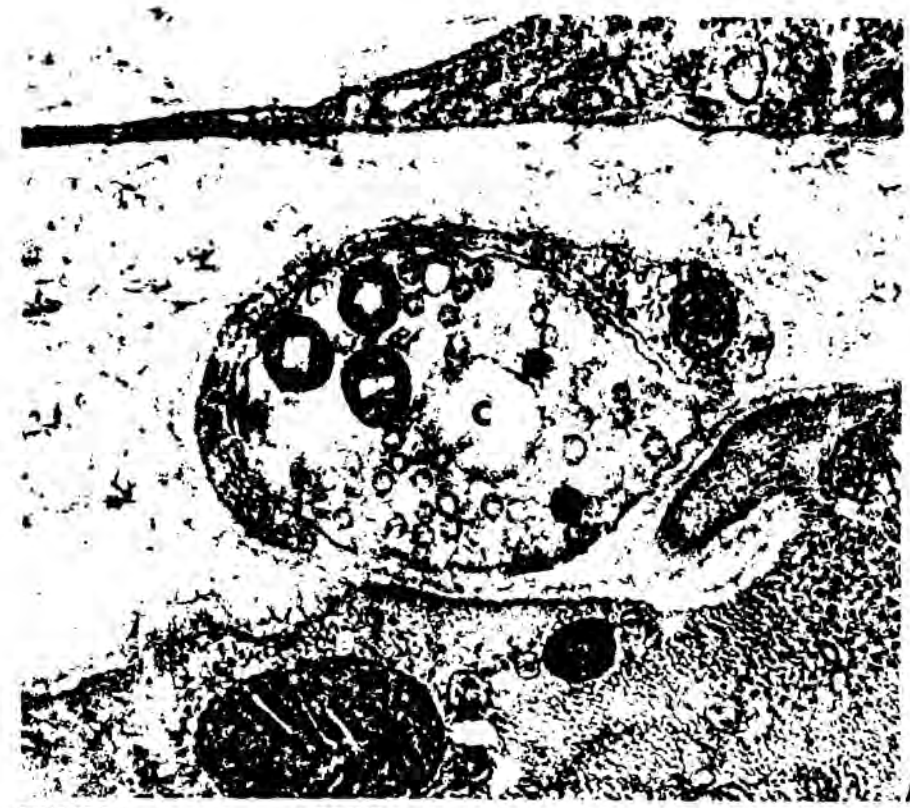
Figure 10. Electron micrographs of magnesium Ringer's control and stimulated nerve terminal cross-sections.

Rapid-frozen control nerve terminals from juvenile Rana pipiens in 10 mM Mg Ringer's (A) appeared morphologically identical to frozen control nerve terminals in normal (1.8 mM Ca) Ringer's and contained occasional cisternal profiles (C).

In rapid-frozen nerve terminals stimulated in 10 mM Mg Ringer's (B) the number of synaptic vesicles was slightly lower and the amount of membrane in cisternae (C) higher. Synaptic vesicle diameter appeared to increase. (magnification = 70,500X)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per nerve terminal profile.

The most striking result of stimulating preparations in high calcium Ringer's as opposed to normal Ringer's was a relatively enormous increase in the number of cisternal profiles per terminal cross-section (Figures 19C and 19D). The total amount of cisternal membrane in these terminals increased, therefore (Figure 20), even though the average cisternal size did not change (Figures 19C and 19D). Relative to changes seen after stimulation in the normal Ringer's group, the percentage of synaptic vesicle number reduction was approximately the same (Figure 18). However, the number of synaptic vesicles in the calcium control terminals was the lowest of any of the controls and decreased after stimulation more than any other frozen groups examined (Figures 12 and 18). Increases in vesicle diameter (from 41.8 $\pm$ .0004 (SE) to 43.4 $\pm$ .0002 (SE) ($p < .001$), Table III) were no greater than in the normal Ringer's series.

The only mitochondrial change that was significant in the terminals stimulated in high calcium Ringer's solution was an increase in the total cross-sectional area (with respect to terminals soaked but unstimulated in high calcium). It had been expected that all of the mitochondrial changes would be more pronounced in terminals stimulated in high calcium Ringer's solution but this was not found to be the case.

A few changes were seen between control groups soaked in high magnesium Ringer's solution and those stimulated in high magnesium Ringer's solution (Figures 10A and 10B). The number of synaptic vesicles was lower in the high magnesium controls than in the normal Ringer's controls and diminished slightly with stimulation (Figure 19). The total amount of cisternal membrane (ie., total cisternal circumference) increased slightly after stimulation (Figure 20) though not to levels reached with stimulation in normal or calcium Ringer's solutions. Indications of a reduction in mitochondrial profiles were absent (Figures 21C and 21F; Table III). An unusual finding was that the average synaptic vesicle diameter increased to its highest level in any frozen group, from 43 $\pm$ .0003 (SE) to 45 $\pm$.0001 (SE) ($p < .001$) (Table III).

The pattern of DCV loss in the terminals stimulated in high calcium was similar to that in the first set of experiments (by 56%, Table III) but was unusual in the normal and magnesium sets. The counts of DCVS appeared abnormally low in these control groups and their numbers increased after stimulation, instead of the decrease seen in all other stimulated groups (Tables II and III).

The response of microtubules was inconsistent in the first and second sets of experiments (Tables II and III). The number of microtubules decreased after

stimulation in the first set of experiments. In all control groups of the second set, the numbers of microtubules were low and increased after stimulation in normal and magnesium Ringer's solutions (to the control levels of the first set). No change in microtubule number was seen with stimulation in high calcium Ringer's solution.

III. Hemicholinium experiments

Comparisons were made between control muscles soaked in normal Ringer's solution and control muscles soaked in 100 μM HC-3. Soaking in HC-3 affected the morphology of the presynaptic nerve terminals (Table IV). The number of synaptic vesicles decreased 41% in HC-3 (Figure 22). This was coupled with decreases in the number of cisternal profiles and the total amount of cisternal membrane per terminal, to levels below those in any other experimental group (Figure 24; Tables II, III and IV).

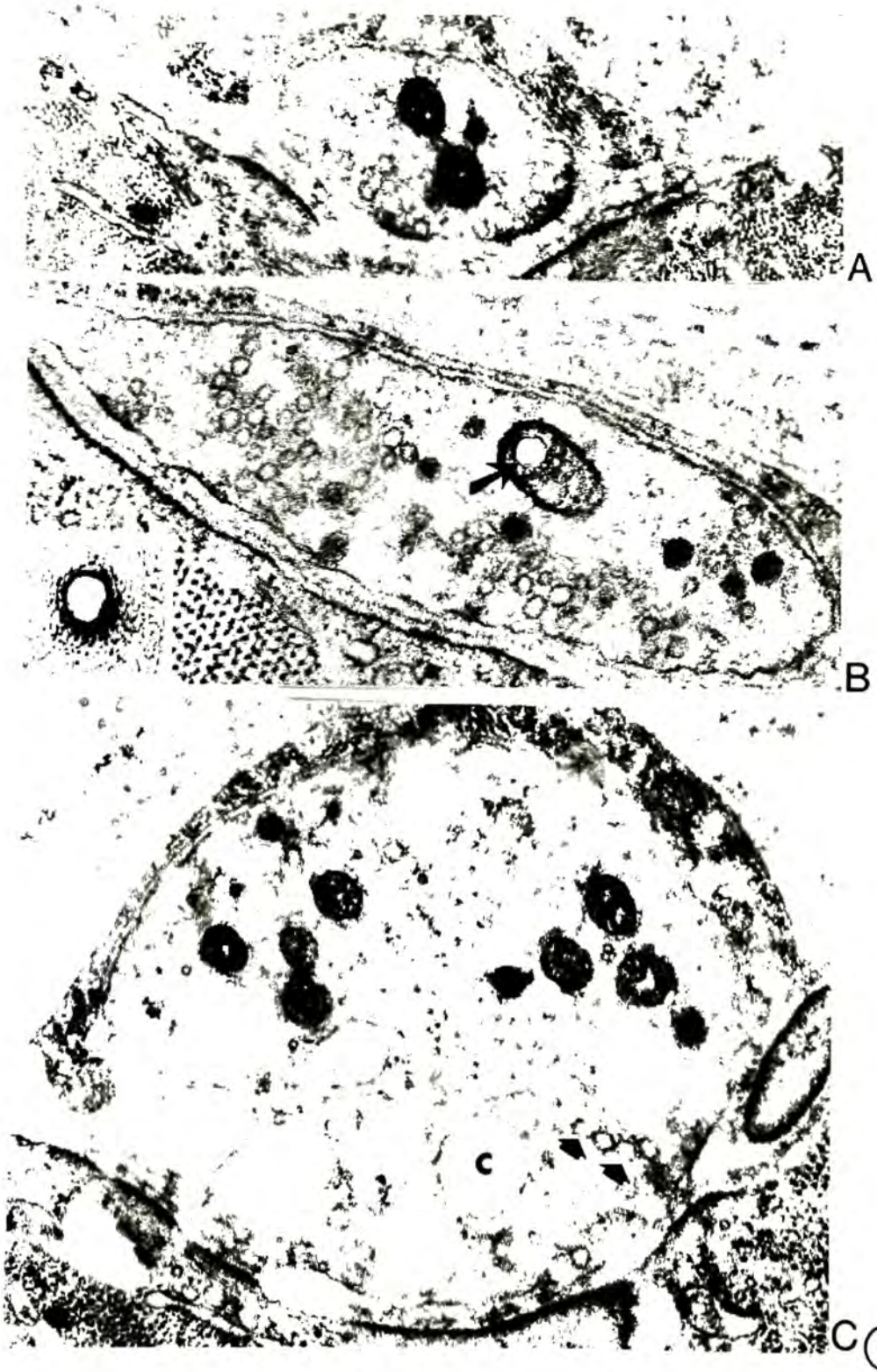
When muscles soaked in HC-3 were stimulated at 15 Hz for 15 minutes, the presynaptic terminals demonstrated most of the effects of stimulation (Figure 11B). The number of synaptic vesicles decreased (by 27% from HC-3 controls, Figure 22). Terminal cross-sectional area and circumference increased relative to the HC-3 controls

Figure 11. Electron micrographs of hemicholinium Ringer's control, stimulated and rested nerve terminal cross-sections.

Rapid-frozen control nerve terminals from juvenile Rana pipiens soaked in 100 μM HC-3 (A) showed differences from all previous rapid-frozen controls. The number of synaptic vesicles and cisternae were decreased from normal control levels.

With stimulation in HC-3 (B) the number of synaptic vesicles decreased from HC-3 control levels. Mitochondria underwent stimulation-induced rounding-up. They often contained large granules or holes (*). (Insert: hole in a postsynaptic mitochondrion.)

Sixty minutes of rest in HC-3 after soaking and stimulation in HC-3 (C) produced an even further reduction in the number of synaptic vesicles (short arrows) and a continued increase in the number and sizes of cisternae (C). (magnification = 63,000X)



(11)

though still within the ranges of other frozen terminals, both control and stimulated (Tables II, III and IV). The number of cisternae increased (Figures 23 and 24). Mitochondrial profiles underwent stimulation-induced changes, becoming less frequent per cross-section and decreasing in diameter (Table IV). Of special note was the presence of holes and electron-dense granules in many of the mitochondria after stimulation in HC-3 (Figure 11b). They were also present in the postsynaptic muscle mitochondria (Figure 11B, insert).

With sixty minutes of rest in HC-3 after stimulation, the presynaptic terminals showed no recovery to control morphology (Figure 11C). Synaptic vesicle numbers were further reduced (51% from HC-3 controls (Figure 22)) and the number and total area of cisternal profiles were greater than in the terminals stimulated in HC-3 (Figures 23C and 24).

TABLES

TABLE II

STATISTICS OF THE FIRST SET OF EXPERIMENTS

	(A) FIX CONT	(B) FIX STIM	(C) FIX REST	(D) FROZ CONT	(E) FROZ STIM	(F) FROZ REST
Terminal area (μm^2)	1.02*	1.45_A	$1.20_{a,B}$	$.71_A$	$.71_B$	$.75_C$
	.122	.110	.113	.063	.076	.054
	(4)	(74)	(50.6)	(33.3)	(4)	(4)
Terminal circumference (μm)	4.88	6.68_A	$5.63_{A,B}$	4.09_A	4.10_B	$3.50_{C,D,E,}$
	.389	.401	.313	.260	.448	.131
	(4)	(57.3)	(74)	(74)	(4)	(26.7)
Number of synaptic vesicles per terminal cross-section	67.70	47.27_A	60.89_B	60.37	40.51_D	58.09_E
	6.006	7.263	6.222	6.037	4.476	4.598
	(4)	(4)	(32)	(74)	(4)	(74)
Flat synaptic vesicles (% of total)	1	3_A	1_b	.2	$.6_B$	0
Average round synaptic vesicle diameter (nm)	49.400	50.587	47.959_b	38.645_A	$42.065_{B,D}$	$39.867_{C,e}$
	.0009	.0008	.0014	.0011	.0009	.0009
	(4)	(4)	(4)	(4)	(4)	(4)
Number of dense-cored vesicles per terminal cross-section	.88	.69	.73	1.21_a	.91	$.61_D$
	.106	.168	.229	.129	.160	.144
	(4)	(4)	(4)	(4)	(4)	(4)
Average dense-cored vesicle circumference (μm)	.27	.28	.26	$.21_A$	$.21_{B,d}$	$.22_{C,D,E}$
	.013	.004	.020	.005	.004	.003
	(4)	(4)	(4)	(4)	(67)	(45)
Number of coated vesicles per terminal cross-section	.00	.07	.03	.01	.28	.11
	.000	.037	.016	.013	.182	.075
	(4)	(4)	(4)	(4)	(4)	(4)

Number of cisternal profiles per terminal cross-section	5.04 .923 (4)	6.01_{a} .652 (48.3)	5.52 .821 (4)	2.75_{A} .290 (74)	$8.40_{B,D}$ .822 (4)	$4.73_{D,E}$ .483 (4)
Total cisternal circumference per terminal cross-section (μm)	1.43 .217 (4)	2.57_{a} .581 (4)	1.66 .289 (4)	$.76_{A}$ .083 (74)	2.61_{D} .264 (4)	$1.19_{C,D,E}$ .113 (4)
Flat cisternal profiles (% of total)	30	45	40	20	10	12
Number of mitochondrial profiles per terminal cross-section	4.97 .422 (74)	3.64_{A} .533 (4)	6.27_{A} .540 (51)	5.75_{A} .562 (74)	3.41_{D} .354 (74)	$3.41_{C,D}$ .509 (4)
Total mitochondrial area per terminal cross-section (μm^2)	.093 .0082 (4)	$.357_{A}$ .0365 (74)	$.167_{A,B}$ .0179 (49.2)	$.074_{A}$ .0069 (74)	$.071_{B}$ .0122 (4)	$.055_{C,D}$ .0136 (4)
Average mitochondrial cross-sectional area (μm^2)	.020 .0004 (4)	$.111_{A}$ .0054 (4)	$.027_{A,B}$ .0011 (4)	$.013_{A}$ .0003 (4)	$.023_{B,D}$ .0010 (4)	$.016_{C,D}$ .0006 (4)
Number of microtubules per terminal cross-section	7.04 .815 (4)	3.96_{A} .433 (39)	6.17_{B} .657 (4)	7.17 .857 (4)	$3.16_{B,D}$ .394 (47.9)	$2.99_{C,D}$ .602 (74)

*Mean ± standard error of the mean (degrees of freedom)

Each group is designated by a letter (above group name). Capital letter subscripts indicate groups which differed at $p < .001$; small-case letters subscripts indicate groups which differed at $p < .01$.

FIX=Fixed; FROZ=Frozen; CONT=Control; STIM=Stimulated; REST=Rested

TABLE III

STATISTICS OF THE CALCIUM/MAGNESIUM SET OF EXPERIMENTS

	(G) NM CONT	(H) NM STIM	(I) CA CONT	(J) CA STIM	(K) MG CONT	(L) MG STIM
Terminal area (μm^2)	.81*	.67	.85	.97	.72	.62
	.153	.081	.244	.285	.103	.084
	(1)	(29)	(1)	(1)	(1)	(23.5)
Terminal circumference (μm)	4.07	4.55	3.79	3.99	3.58	3.82
	.542	.441	.290	.361	.309	.371
	(1)	(29)	(29)	(19.3)	(1)	(22.1)
Number of synaptic vesicles per terminal cross-section	58.77_{D}	33.03_{G}	47.10_{G}	25.63_{I}	41.37_{G}	35.13_{K}
	8.148	4.255	5.700	5.504	6.259	4.932
	(29)	(1)	(29)	(1)	(29)	(29)
Average round synaptic vesicle diameter (nm)	40.761	43.670	41.758	43.321	39.710	45.402
	.0012	.0022	.0004	.0001	.0003	.0013
	(1)	(1)	(1)	(1)	(1)	(1)
Number of dense-cored vesicles per terminal cross-section	$.50_{D}$	.60	1.13	.50	.47	.67
	.100	.267	.133	.033	.333	.000
	(1)	(1)	(1)	(1)	(1)	(1)
Average dense-cored vesicle circumference (μm)	$.22_{D}$	$.22_{E}$	.22	.24	$.21_{G}$	$.23_{K}$
	.005	.008	.018	.013	.006	.005
	(14)	(17)	(1)	(1)	(13)	(19)
Number of coated vesicles per terminal cross-section	.07	.10	.03	.17	.00	.13
	.067	.033	.033	.167	.000	.067
	(1)	(1)	(1)	(1)	(1)	(1)

Number of cisternal profiles per terminal cross-section	3.40_{D} .744 (29)	3.23_{E} .507 (29)	2.90 .766 (1)	8.10_{I} 1.360 (20.1)	2.27_{G} .368 (29)	1.81 .423 (1)
Total cisternal circumference per terminal cross-section (μm)	.82 .186 (29)	$1.15_{E,G}$ .214 (29)	.62 .337 (1)	3.85_{I} .640 (22.9)	$.53_{G}$ .112 (20)	$.69_{K}$ .138 (29)
Flat cisternal profiles (% of total)	20	10	15	5	10	15
Number of mitochondrial profiles per terminal cross-section	2.70_{D} 1.233 (1)	2.20_{E} .467 (1)	2.65 .501 (21.5)	1.89 .686 (1)	2.47 .348 (29)	2.70 .375 (29)
Total mitochondrial area per terminal cross-section (μm^2)	$.039_{D}$ .0079 (1)	.059 .0168 (1)	.034 .0077 (1)	$.061_{i}$ .0134 (29)	.034 .0156 (1)	.043 .0051 (19.9)
Average mitochondrial cross-sectional area (μm^2)	.013 .0013 (1)	$.033_{e}$ .0039 (1)	.013 .0014 (1)	.034 .0053 (1)	.015 .0015 (1)	.017 .0009 (1)
Number of microtubules per terminal cross-section	$.74_{D}$ .502 (1)	$7.66_{E,G}$ 1.908 (22.7)	.93 .467 (1)	1.43 .457 (29)	1.53 .492 (1)	5.24_{K} 1.138 (20)

*Mean ± standard error of the mean (degrees of freedom)
Each group is designated by a letter (above group name). Capital letter subscripts indicate groups which differed a $p < .001$; small-case letter subscripts indicate groups which differed at $p < .01$.
NM=Normal; CA=Calcium; MG=Magnesium

TABLE IV

STATISTICS OF THE HEMICHOLINIUM SET OF EXPERIMENTS

	(M) HC CONT	(N) HC STIM	(O) HC REST
Terminal area (μm^2)	.56*	$.83_M$	.77
	.117	.071	.125
	(2)	(44)	(2)
Terminal circumference (μm)	2.85	4.50_m	3.82_M
	.450	.415	.337
	(2)	(2)	(44)
Number of synaptic vesicles per terminal cross-section	35.38_G	25.73_M	16.63_M
	4.112	3.706	2.274
	(44)	(2)	(25)
Average round synaptic vesicle diameter (nm)	43.987	43.107	44.832
	.0013	.0008	.0009
	(3)	(4)	(4)
Number of dense-cored vesicles per terminal cross-section	.67	1.00	.73
	.139	.467	.168
	(2)	(2)	(2)
Average dense-cored vesicle circumference (μm)	.22	.22	.23
	.005	.004	.011
	(29)	(2)	(2)
Number of coated vesicles per terminal cross-section	.00	.20	.00
	.000	.039	.000
	(2)	(2)	(2)

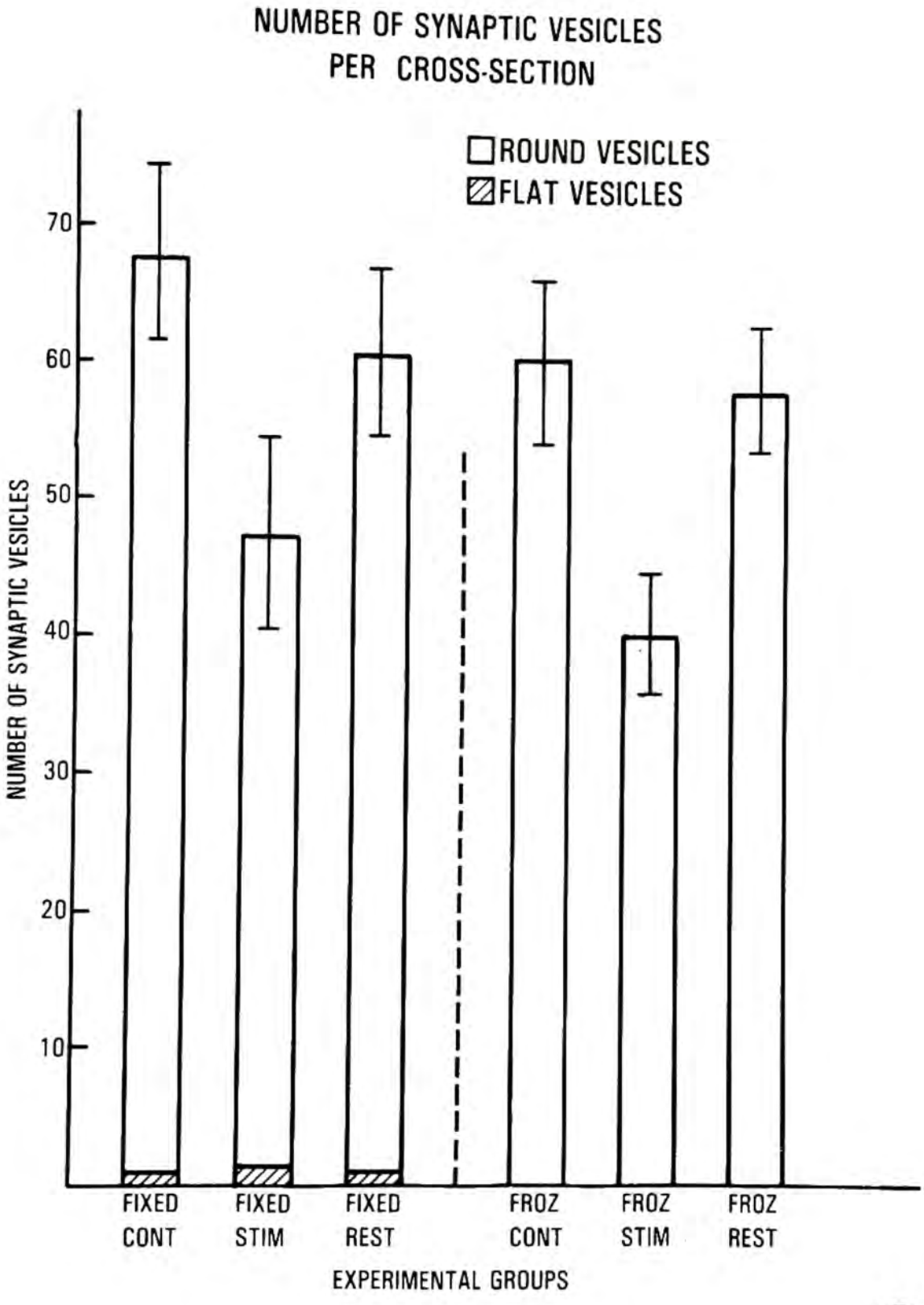
Number of cisternal profiles per terminal cross-section	1.15_G .244 (32)	5.67_M .661 (44)	7.11_M 1.308 (2)
Total cisternal circumference per terminal cross-section (μm)	$.30_G$ .070 (2)	1.77_M .218 (44)	2.92_m .599 (2)
Flat cisternal profiles (% of total)	10	10	10
Number of mitochondrial profiles per terminal cross-section	4.89 .739 (2)	2.82_M .323 (44)	2.96_M .524 (44)
Total mitochondrial area per terminal cross-section (μm^2)	.029 .0052 (32)	$.063_M$ .0169 (2)	$.056_M$ .0107 (44)
Average mitochondrial cross-sectional area (μm^2)	.015 .0008 (92)	$.025_M$ .0023 (2)	$.019_M$ .0019 (2)
Number of microtubules per terminal cross-section	.70 .400 (22.2)	7.16_M 1.012 (2)	2.26_M .688 (33.4)

*Mean ± standard error of the mean (degrees of freedom)
Each group is designated by a letter (above group name). Capital letter subscripts indicate groups which differed at $p < .001$; small-case letter subscripts indicate groups which differed at $p < .01$
HC=Hemicholinium

FIGURES

Figure 12. A histogram of the number of synaptic vesicles per nerve terminal cross-section for the first set of experiments.

Each bar represents the mean number of synaptic vesicles (±SE) per nerve terminal cross-section in each of the six groups of the first set of experiments. Each mean was calculated from the mean number of synaptic vesicles per nerve terminal cross-section for each of the five muscles in the experimental group. In both the chemically fixed and rapid-frozen groups, the number of synaptic vesicles per nerve terminal decreased from control values with stimulation. Following a 60 minute rest period, both chemically fixed and rapid-frozen nerve terminals showed an increase in the number of synaptic vesicles back to control levels. In the chemically fixed groups an increase in the number of flat synaptic vesicles was observed after stimulation, with a subsequent fall to control values after rest. (Only differences that were significant at $p < .01$ are discussed. See Table II for statistics.)


NUMBER OF SYNAPTIC VESICLES
PER CROSS-SECTION
ROUND VESICLES
FLAT VESICLES
NUMBER OF SYNAPTIC VESICLES
70
60
50
40
30
20
10
FIXED CONT
FIXED STIM
FIXED REST
FROZ CONT
FROZ STIM
FROZ REST
EXPERIMENTAL GROUPS

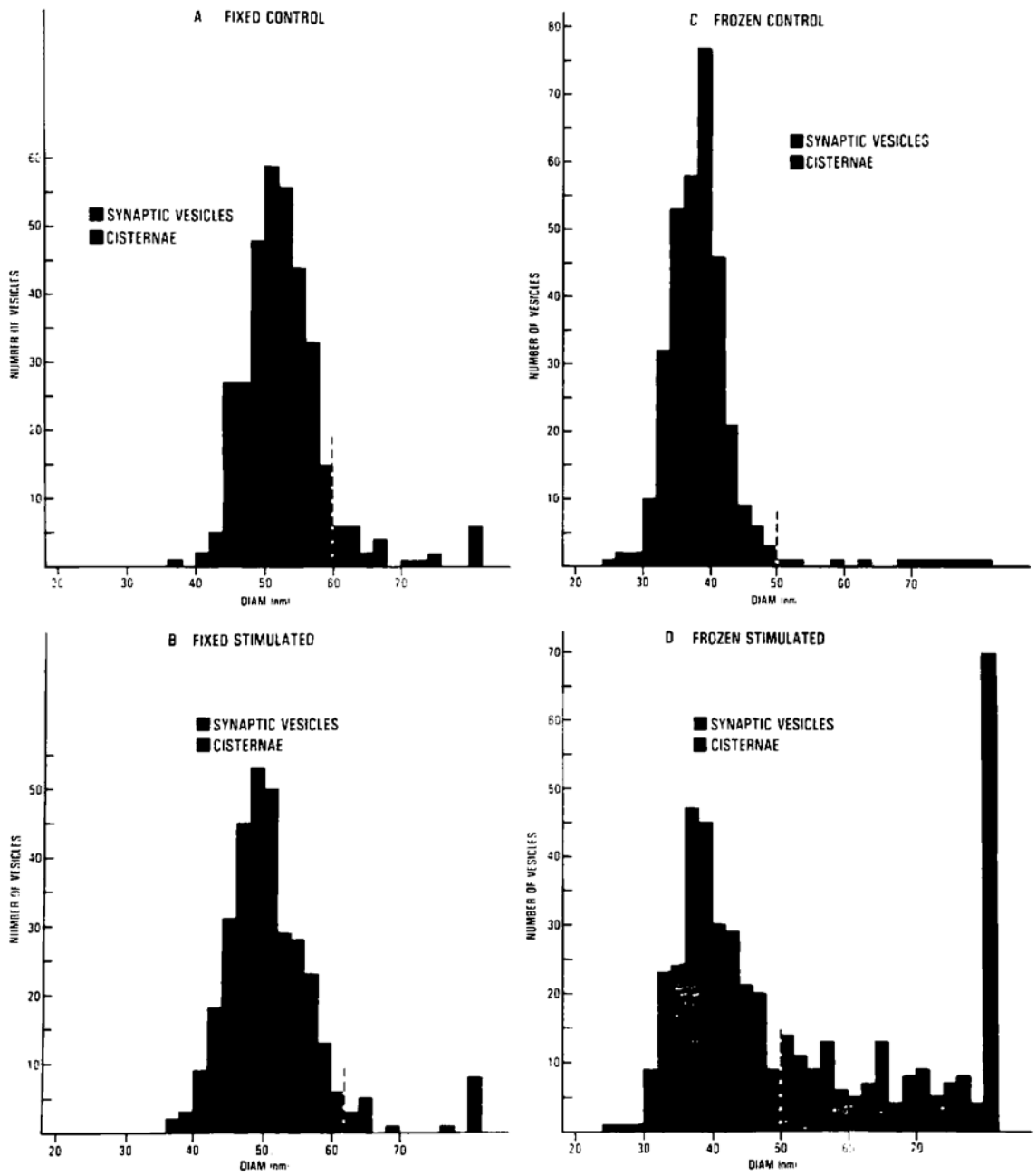
(12)

Figure 13. Histograms of the distribution of vesicle diameters for the first set of experiments.

Each graph contains data from a single muscle representative of each of four experimental groups. (Rested groups were not included because their distribution was identical to the controls.) To produce each histogram, the circumference and diameter of all vesicular profiles were measured on enough micrographs (from 2-15) to accumulate 300-500 measurements for each muscle. These values were then plotted to determine a cutoff point between synaptic vesicles and cisternae. Mean synaptic vesicle diameter was then computed for all profiles to the left of the cutoff.

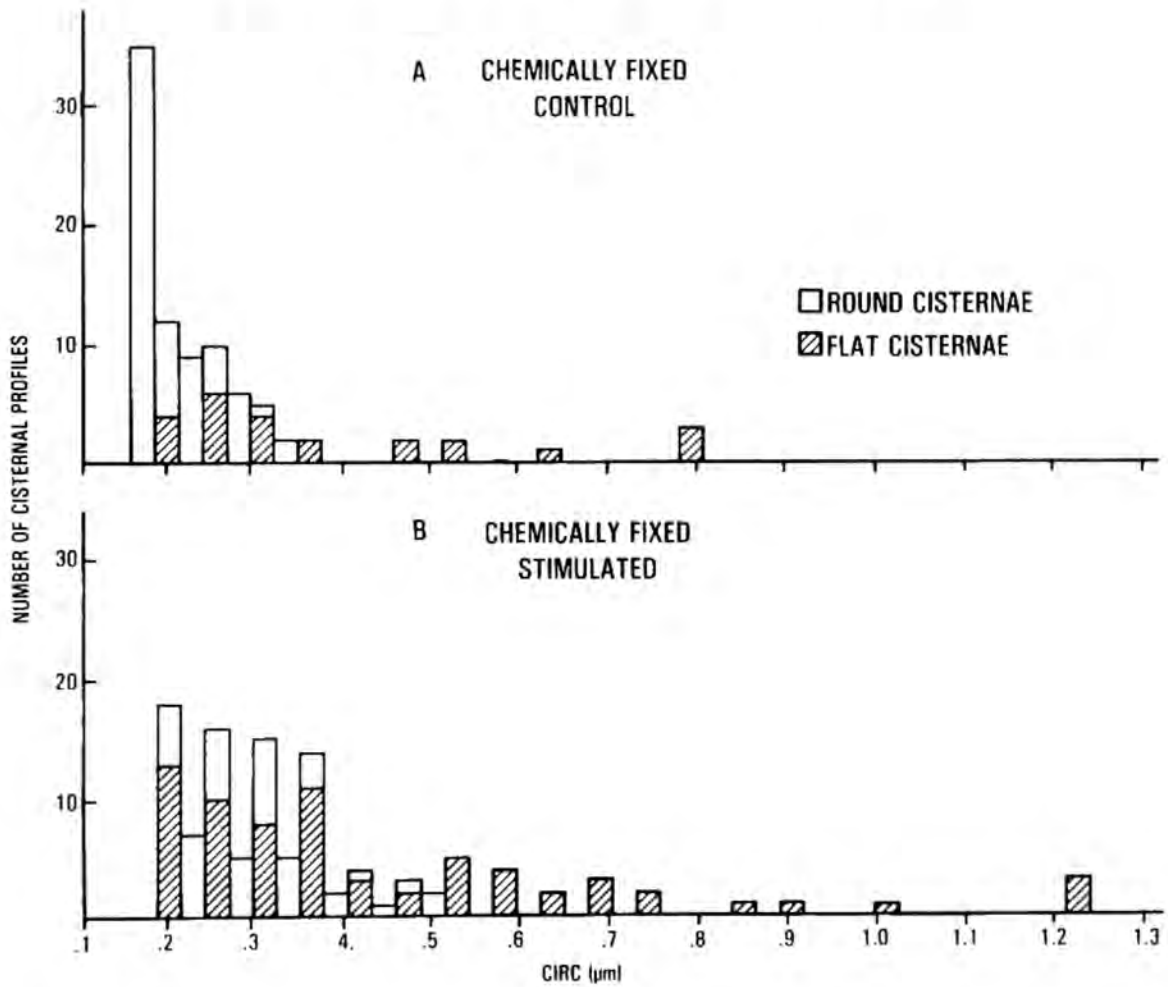
Synaptic vesicles in chemically fixed control nerve terminals (A) had a mean diameter of 49.4 nm. Synaptic vesicles in fixed stimulated nerve terminals (B) averaged 50.6 nm. diameter. Synaptic vesicles in rapid-frozen control nerve terminals (C) had a mean diameter of 38.6 nm. These synaptic vesicles were significantly smaller than those in the fixed controls. Frozen stimulated nerve terminals (D) contained synaptic vesicles with an average diameter of 42.1 nm. These synaptic vesicles were still smaller than those in the chemically fixed nerve terminals.

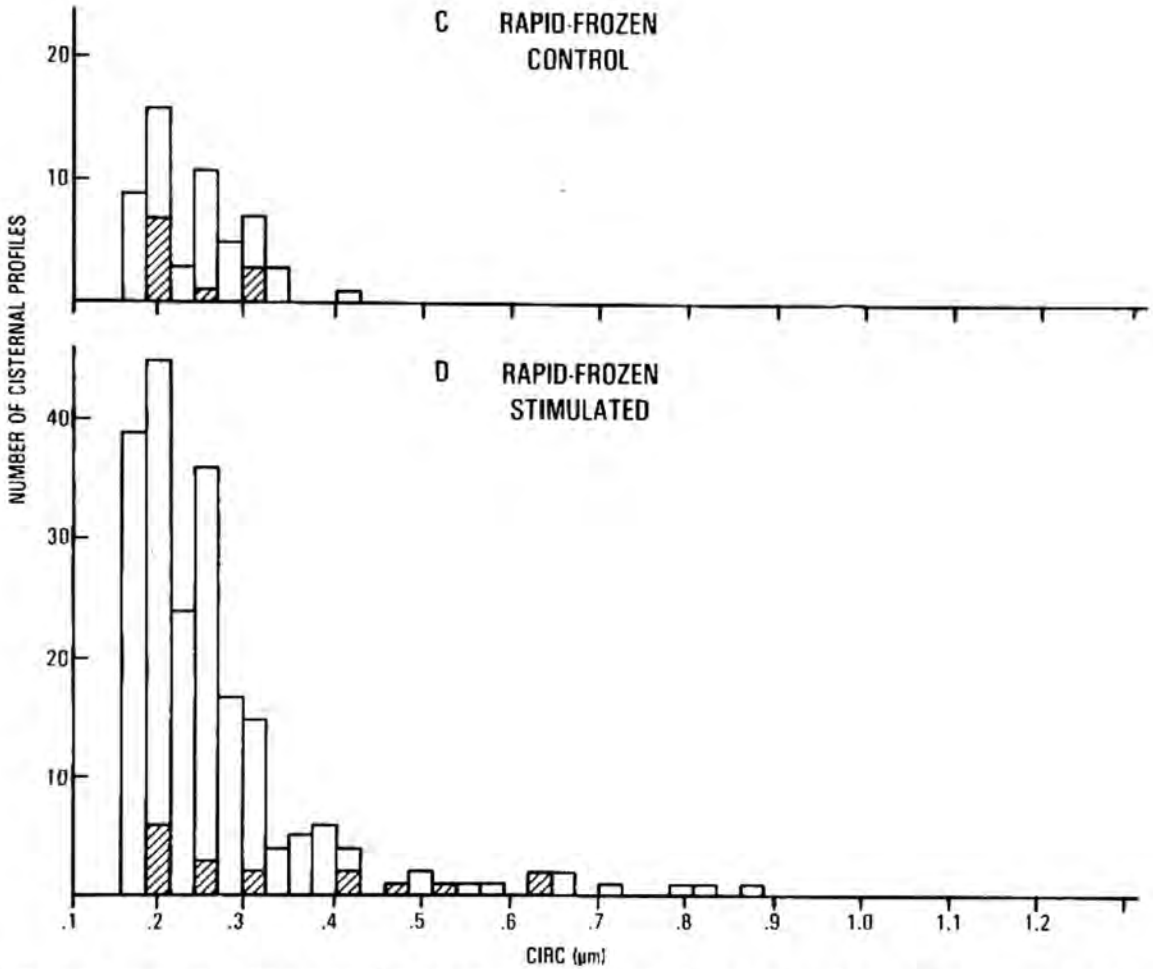
DISTRIBUTION OF SYNAPTIC VESICLE DIAMETERS



13

DISTRIBUTION OF CISTERNAL CIRCUMFERENCES





14

Figure 14. Histograms of the distribution of cisternal circumferences for the first set of experiments. These graphs contain all measurements from a representative muscle from four groups. Fixed controls (A) had more round cisternae than flat but the flat ones were often larger. After stimulation (B) the number of cisternae was slightly higher. The total amount of membrane also increased, with the flat cisternae larger than in the controls. Frozen controls (C) had few cisternae. They were usually round and small. The number increased after stimulation (D). More round than flat cisternae were observed and larger examples of both types appeared.

Figure 15. A histogram of the total cisternal circumference per nerve terminal cross-section for the first set of experiments.

Each bar represents the mean amount of cisternal membrane (±SE) per nerve terminal cross-section in each of the six experimental groups of the first set of experiments. Each mean was calculated from the mean total cisternal circumference per nerve terminal cross-section for all five muscles in each experimental group.

The amount of cisternal membrane per nerve terminal (from both round and flat cisternae) was greater after stimulation in both the chemically fixed and rapid-frozen terminals. Fixed control nerve terminals had more membrane than frozen controls, but both increased to the same amount after stimulation. With rest, the amount of cisternal membrane decreased in both groups to within their control ranges.

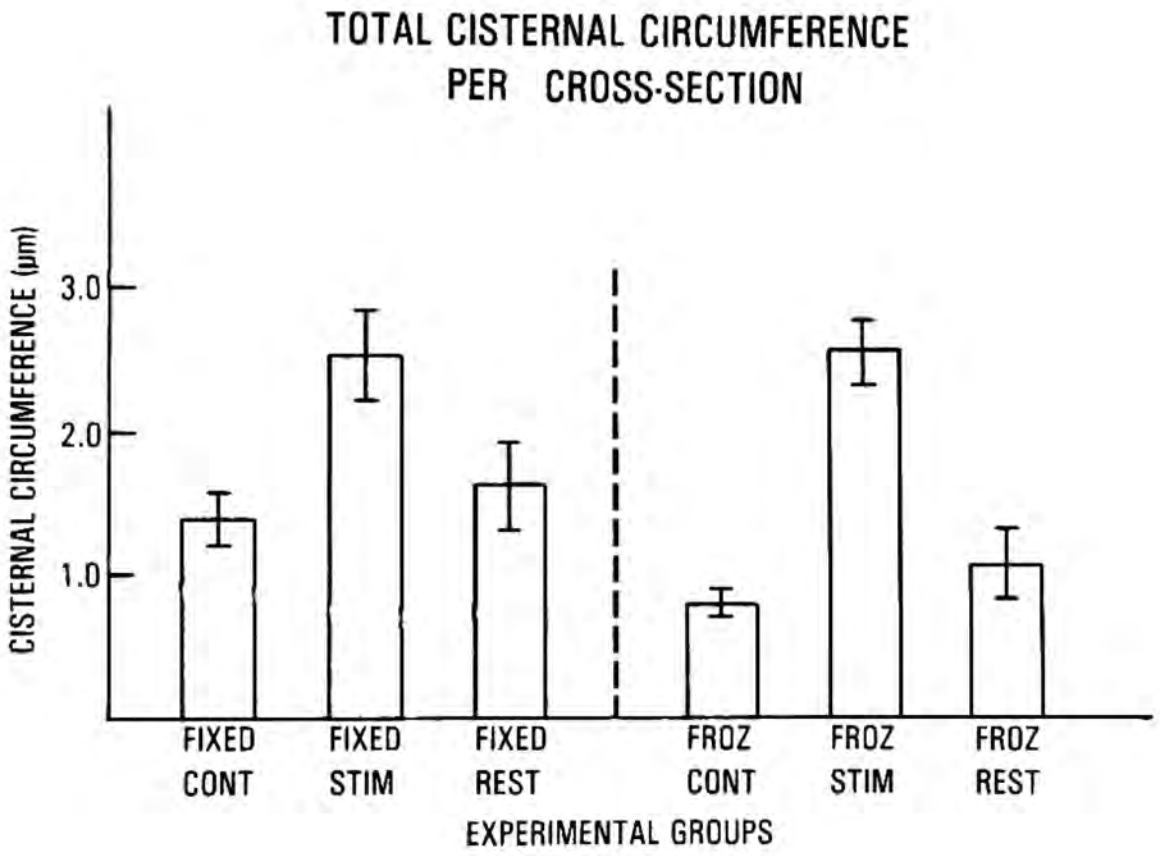
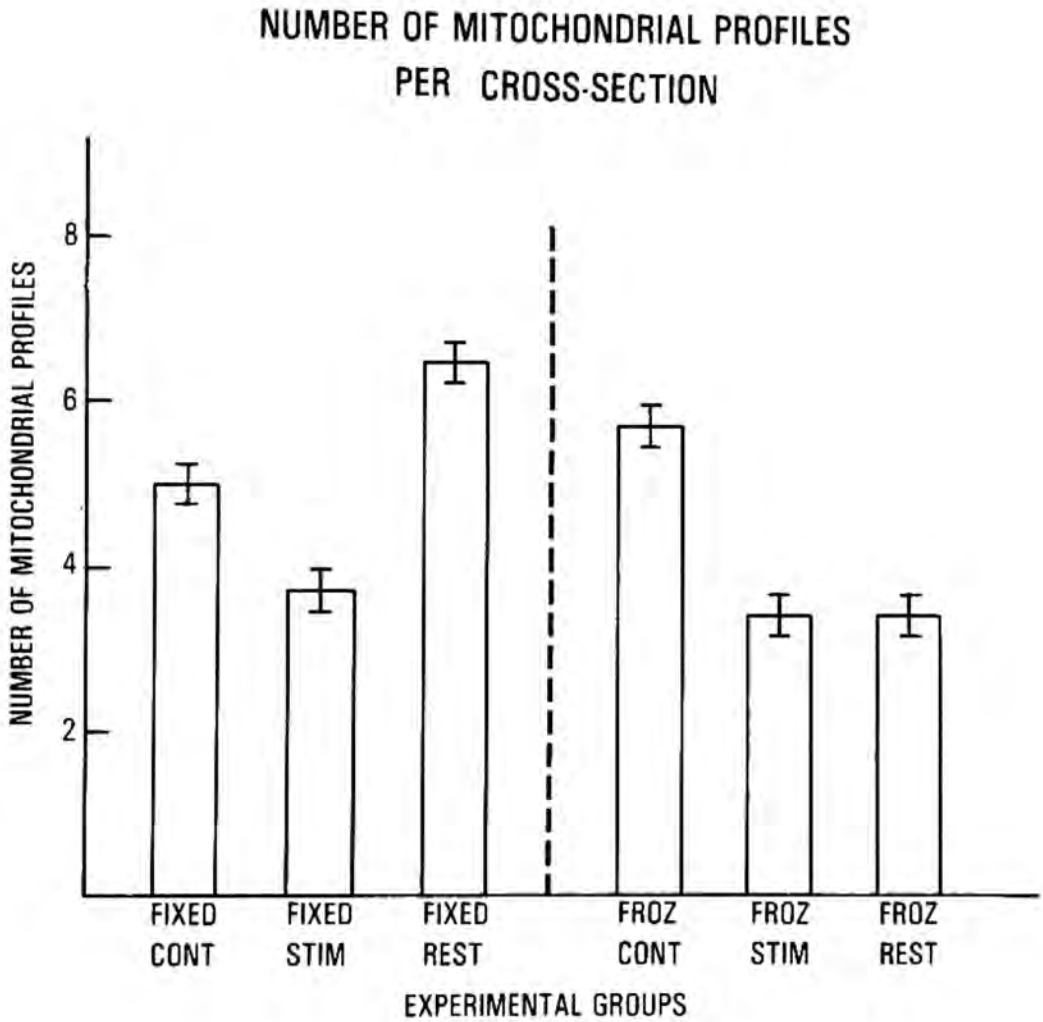
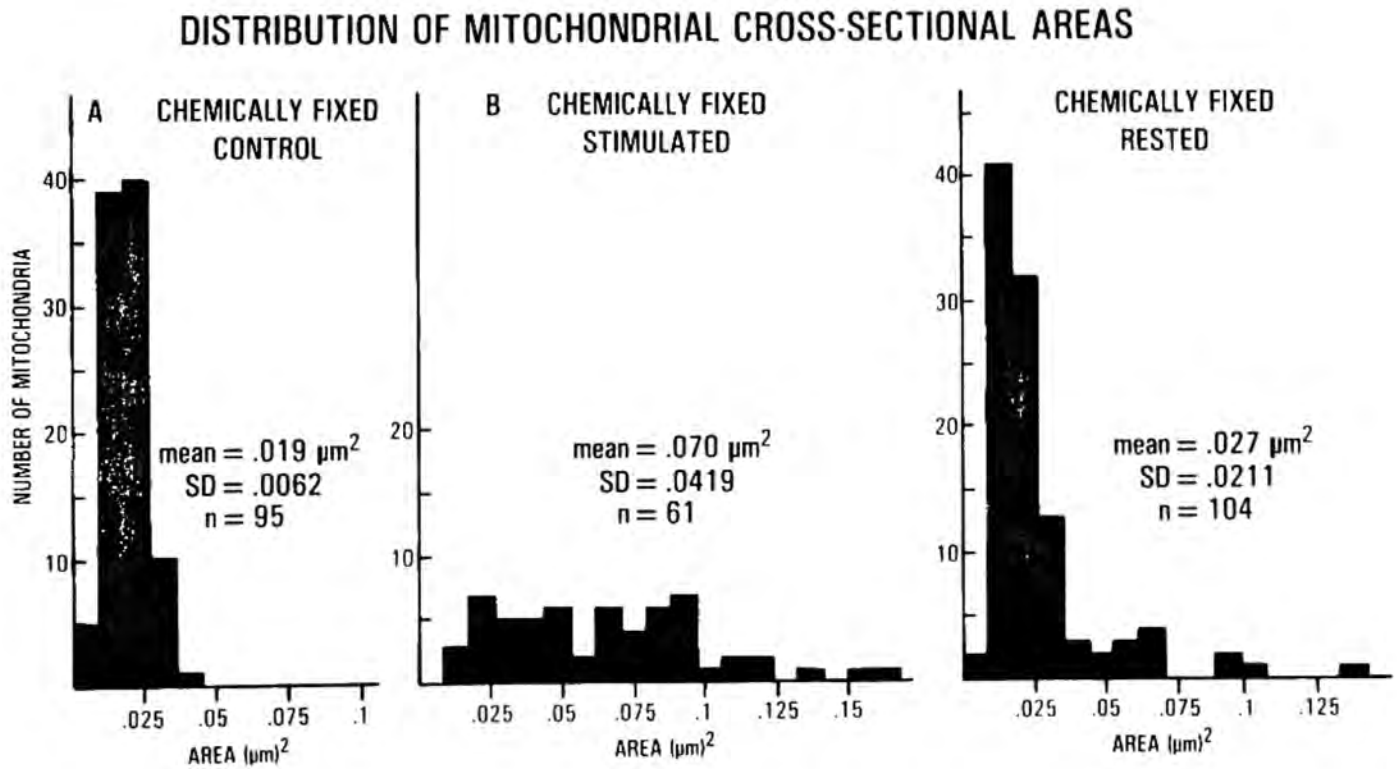

TOTAL CISTERNAL CIRCUMFERENCE
PER CROSS-SECTION
CISTERNAL CIRCUMFERENCE (µm)
3.0
2.0
1.0
FIXED CONT
FIXED STIM
FIXED REST
FROZ CONT
FROZ STIM
FROZ REST
EXPERIMENTAL GROUPS

Figure 16. A histogram of the number of mitochondrial profiles per nerve terminal cross-section for the first set of experiments.

Each bar represents the mean number of mitochondrial profiles (±SE) per nerve terminal cross-section in each of the six experimental groups of the first set of experiments. Each mean was calculated from the mean number of mitochondrial profiles per nerve terminal cross-section for all five muscles in each experimental group.

The number of mitochondria in the fixed control groups was less than in the frozen controls. With stimulation the number of mitochondria in both groups decreased to similar levels. With rest the fixed mitochondria appeared to surpass the numbers seen in fixed controls, while the mitochondria in the frozen rested nerve terminals remained diminished.


NUMBER OF MITOCHONDRIAL PROFILES
PER CROSS-SECTION
NUMBER OF MITOCHONDRIAL PROFILES
8
6
4
2
FIXED CONT
FIXED STIM
FIXED REST
FROZ CONT
FROZ STIM
FROZ REST
EXPERIMENTAL GROUPS


DISTRIBUTION OF MITOCHONDRIAL CROSS-SECTIONAL AREAS
A
CHEMICALLY FIXED
CONTROL
mean = .019 μm²
SD = .0062
n = 95
B
CHEMICALLY FIXED
STIMULATED
mean = .070 μm²
SD = .0419
n = 61
CHEMICALLY FIXED
RESTED
mean = .027 μm²
SD = .0211
n = 104
NUMBER OF MITOCHONDRIA
AREA (μm)²
AREA (μm)²
AREA (μm)²

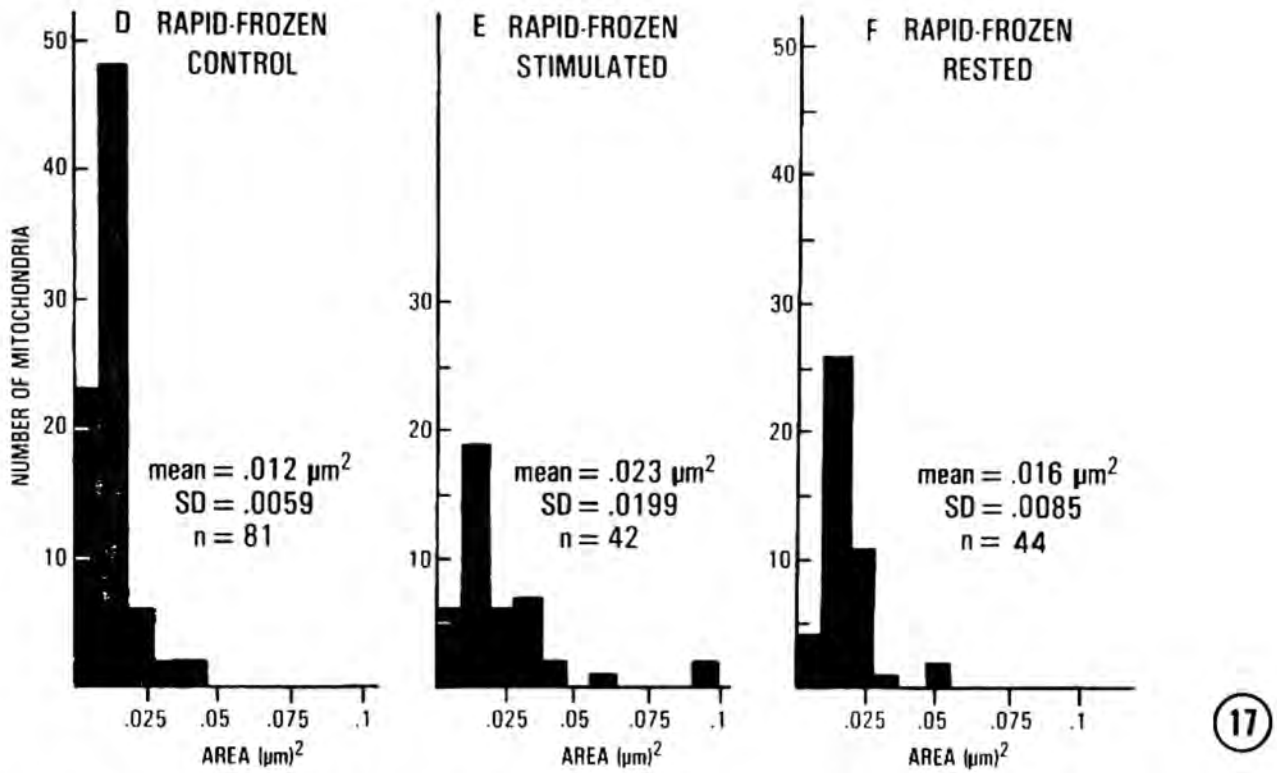
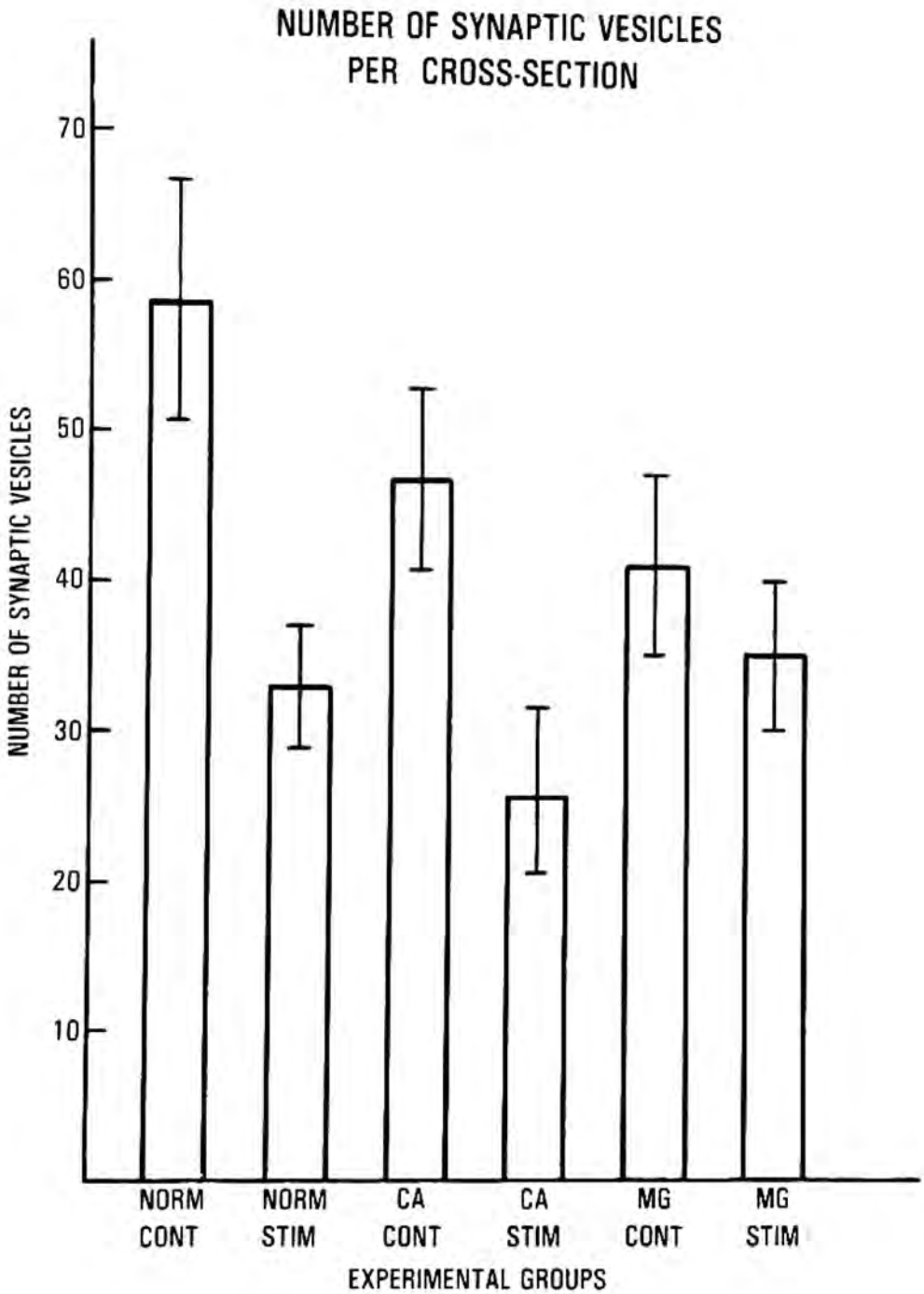


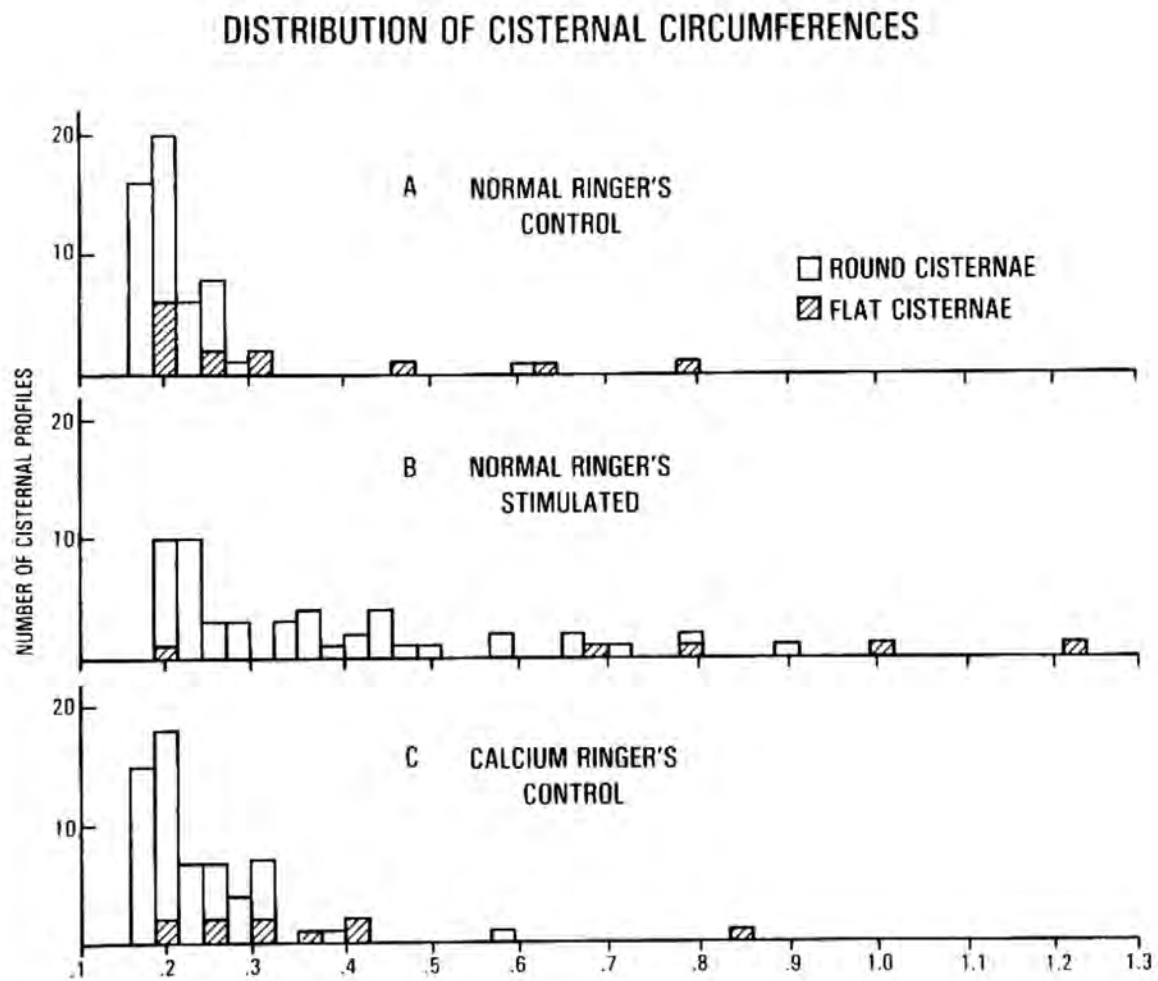
Figure 17. Histograms of the distribution of mitochondrial cross-sectional areas for the first set of experiments. Each graph contains measurements of all mitochondrial profiles from a representative muscle in each of the six groups. Mitochondria in fixed controls (A) were uniform in girth with frozen control mitochondria (D) slightly narrower. After stimulation, mitochondria appeared less frequently in profiles of fixed nerve terminals (ie. they were shorter; they were also swollen.) In frozen nerve terminals (E) they were shorter and rounder but not swollen. Mitochondria in fixed rested terminals (C) were still slightly swollen and their frequency was greater. After rest, mitochondria in frozen terminals (F) remained slightly rounded-up.

Figure 18. A histogram of the number of synaptic vesicles per nerve terminal cross-section for calcium/magnesium series.

Each bar represents the mean number of synaptic vesicles (±SE) per nerve terminal cross-section in each of the six experimental groups in the calcium/magnesium series. Each mean was calculated from the mean number of synaptic vesicles per nerve terminal cross-section for both muscles in each experimental group. The number of synaptic vesicles per nerve terminal cross-section decreased with stimulation in each of the three Ringer's types, with the least reduction in number observed in the 10 mM magnesium group. Both 10 mM calcium control and 10 mM magnesium control groups had fewer synaptic vesicles than the normal control group. (For statistics see Table III.)


NUMBER OF SYNAPTIC VESICLES
PER CROSS-SECTION
NUMBER OF SYNAPTIC VESICLES
70
60
50
40
30
20
10
NORM CONT
NORM STIM
CA CONT
CA STIM
MG CONT
MG STIM
EXPERIMENTAL GROUPS

18


DISTRIBUTION OF CISTERNAL CIRCUMFERENCES
NUMBER OF CISTERNAL PROFILES
20
10
A NORMAL RINGER'S CONTROL
ROUND CISTERNAE
FLAT CISTERNAE
B NORMAL RINGER'S STIMULATED
C CALCIUM RINGER'S CONTROL
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3

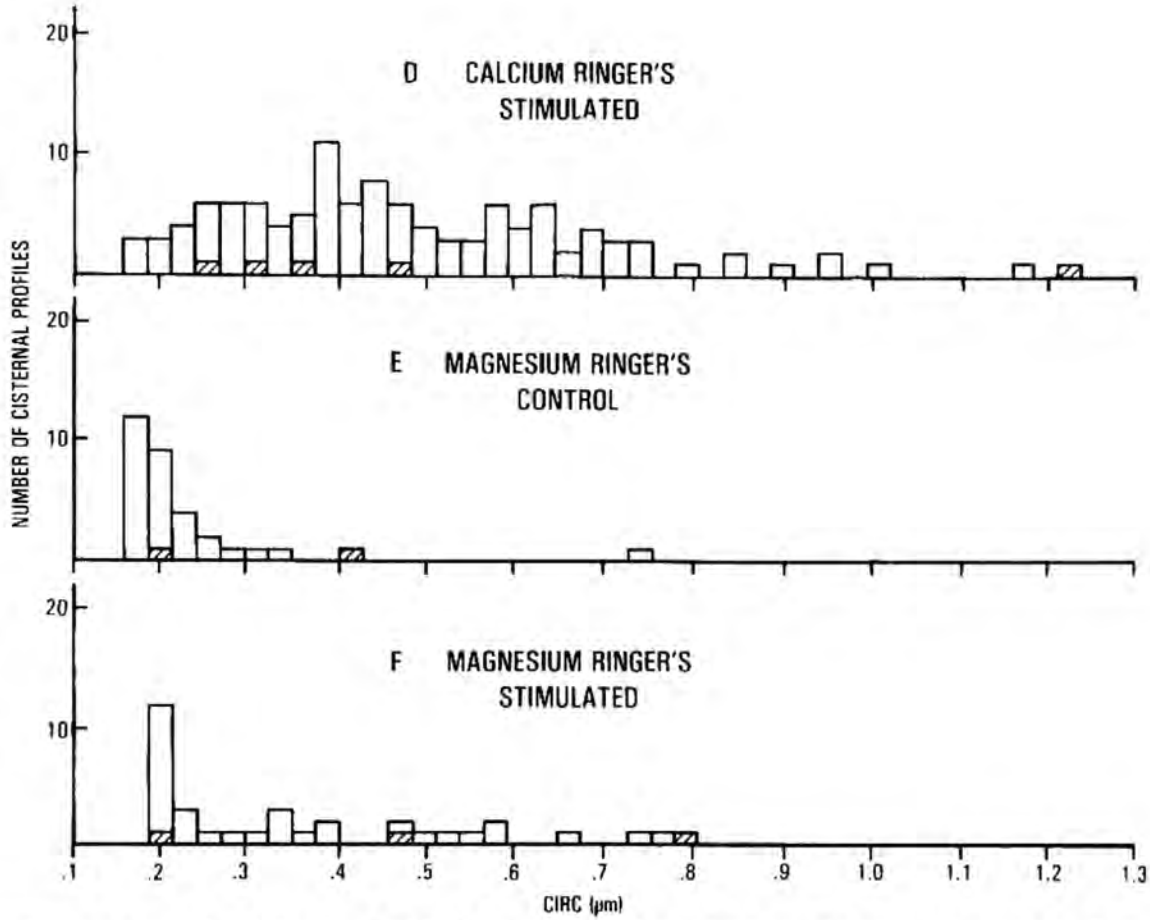


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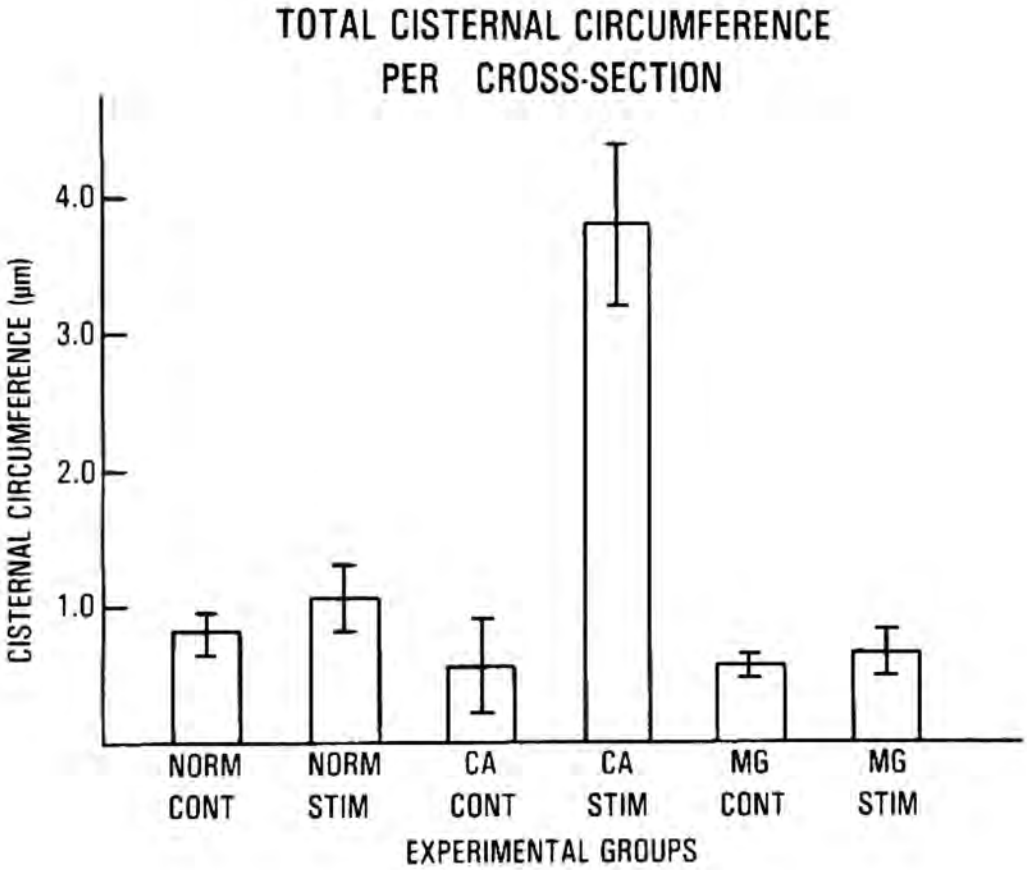
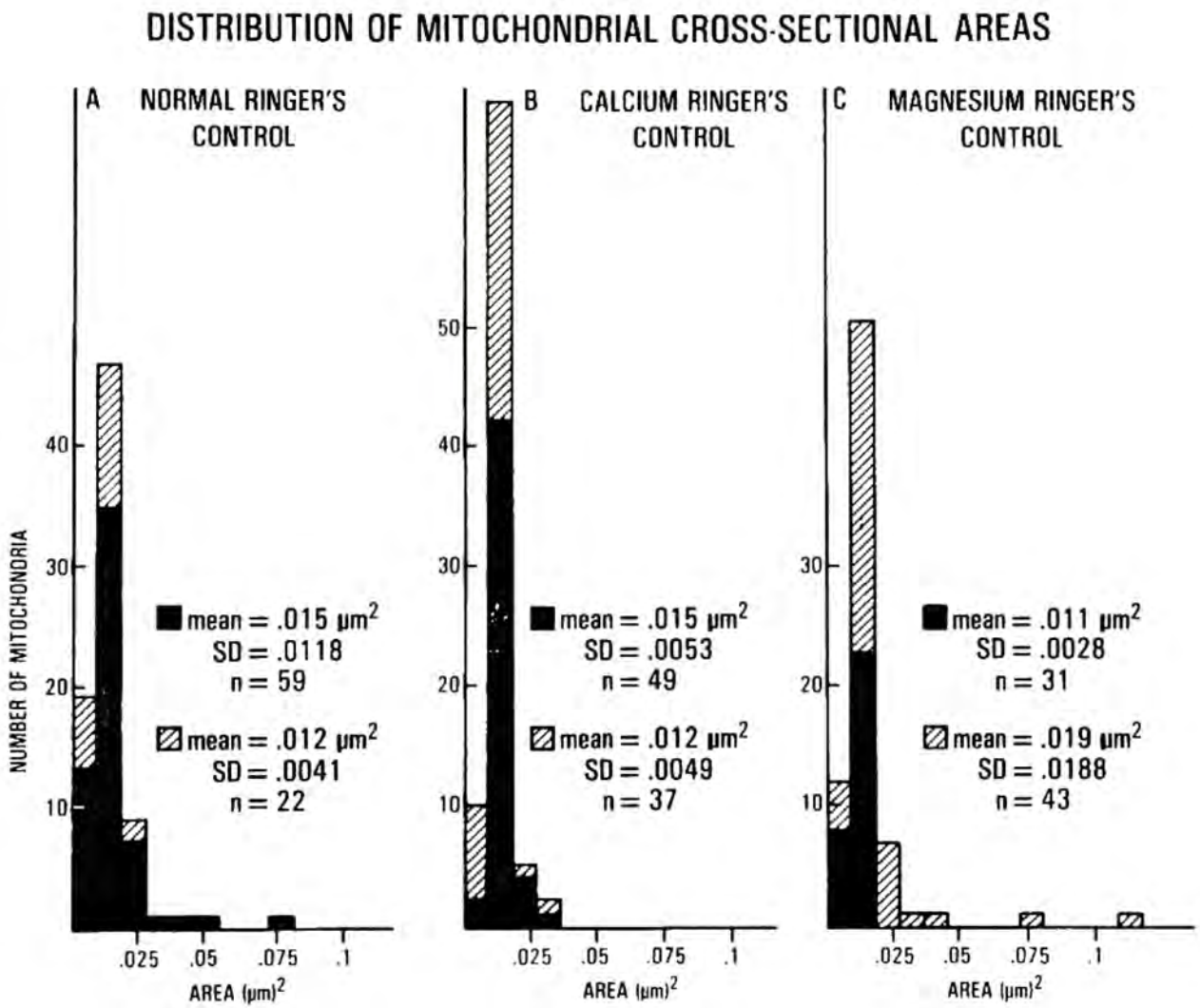
Figure 19. Histograms of the distribution of cisternal circumferences for calcium/magnesium series. These graphs contain all measurements from a representative muscle in each group in the calcium/magnesium series. A normal Ringer's control (A) contained a few small cisternae as did calcium and magnesium controls (C,E). After stimulation in normal Ringer's, (B) the number was not increased but the amount of membrane per profile was. With stimulation in calcium Ringer's (D) the number of cisternae increased dramatically, as did the total amount of membrane. With stimulation in magnesium Ringer's (F) the amount of cisternal membrane increased only slightly above control values.

Figure 20. A histogram of the total cisternal circumference per nerve terminal cross-section for the calcium/magnesium series.

Each bar represents the mean amount of cisternal membrane (±SE) per nerve terminal cross-section in each of the six experimental groups of the calcium/magnesium series. Each mean was calculated from the mean total cisternal circumference per nerve terminal cross-section for both muscles in each experimental group. Total cisternal circumference increased only slightly with stimulation in both the normal (1.8 mM Ca) Ringer's and 10 mM magnesium Ringer's groups. An enormous increase was seen, however, after stimulation in 10 mM calcium Ringer's.


TOTAL CISTERNAL CIRCUMFERENCE
PER CROSS-SECTION
CISTERNAL CIRCUMFERENCE (µm)
4.0
3.0
2.0
1.0
NORM CONT
NORM STIM
CA CONT
CA STIM
MG CONT
MG STIM
EXPERIMENTAL GROUPS


DISTRIBUTION OF MITOCHONDRIAL CROSS-SECTIONAL AREAS
A NORMAL RINGER'S CONTROL
B CALCIUM RINGER'S CONTROL
C MAGNESIUM RINGER'S CONTROL
NUMBER OF MITOCHONDRIA
10
20
30
40
50
.025
.05
.075
.1
AREA (μm)²
mean = .015 μm²
SD = .0118
n = 59
mean = .012 μm²
SD = .0041
n = 22
mean = .015 μm²
SD = .0053
n = 49
mean = .012 μm²
SD = .0049
n = 37
mean = .011 μm²
SD = .0028
n = 31
mean = .019 μm²
SD = .0188
n = 43

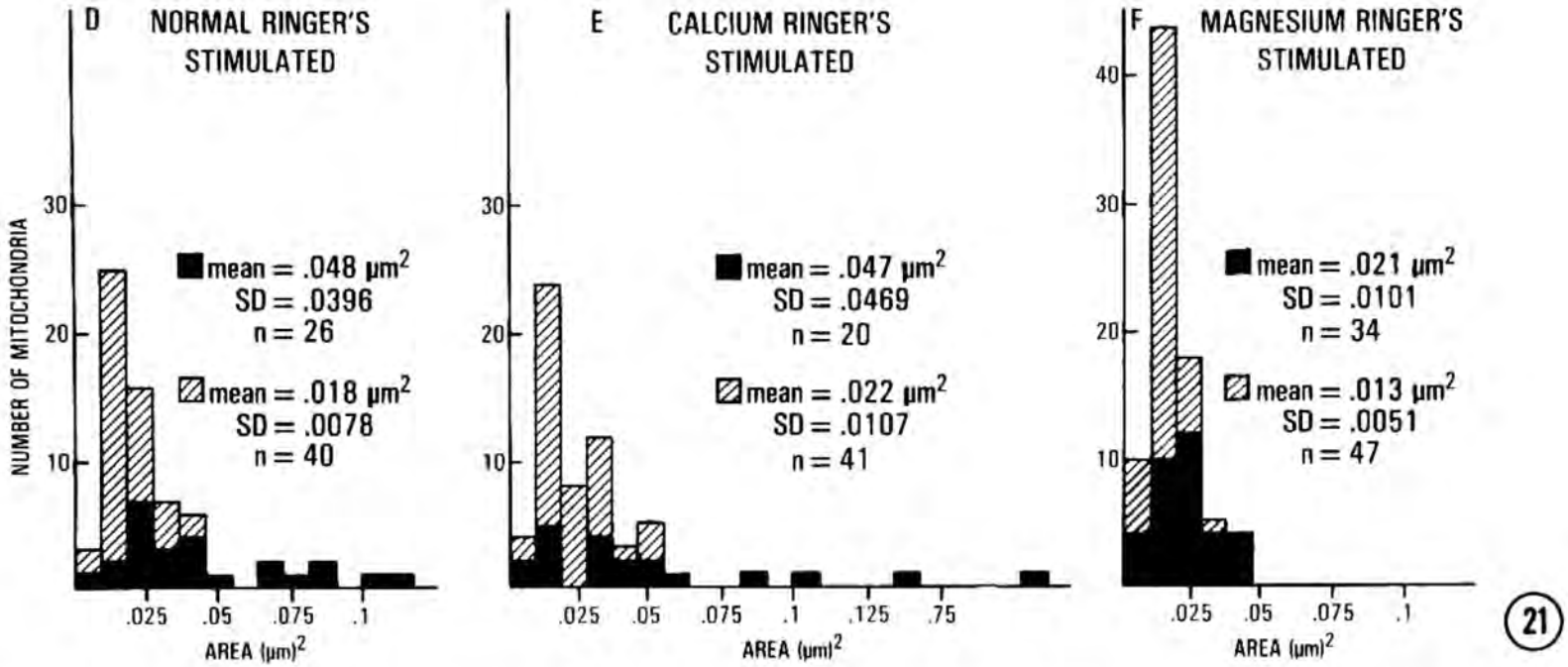


Figure 21. Histograms of the distribution of mitochondrial cross-sectional areas for the calcium/magnesium series. Since neither muscle was representative of the group as a whole, all mitochondrial measurements from both muscles (one dark, one striped) in each of the six experimental groups in the calcium/magnesium series were plotted. Control terminals in all three types of Ringer's (A, B, and C) contained similar numbers of mitochondrial profiles within the same range of cross-sectional area. With stimulation in normal Ringer's (D) and calcium Ringer's (E), mitochondrial profiles dropped in frequency and increased slightly in area, indicating the expected stimulation-induced shape change. (It was expected that the mitochondria in the calcium stimulated group would exhibit greatly exaggerated changes, which they did not.) After stimulation in magnesium Ringer's (F) no indications of mitochondrial shape change were evident.

Figure 22. A histogram of the number of synaptic vesicles per nerve terminal cross-section for the hemicholinium series.

Each bar represents the mean number of synaptic vesicles (±SE) per nerve terminal cross-section in each of the three groups in the hemicholinium series. Each mean was calculated from the mean number of synaptic vesicles per nerve terminal cross-section for all three muscles in each experimental group. A progressive reduction in synaptic vesicle number was observed with stimulation and with subsequent rest. (For statistics see Table IV.)

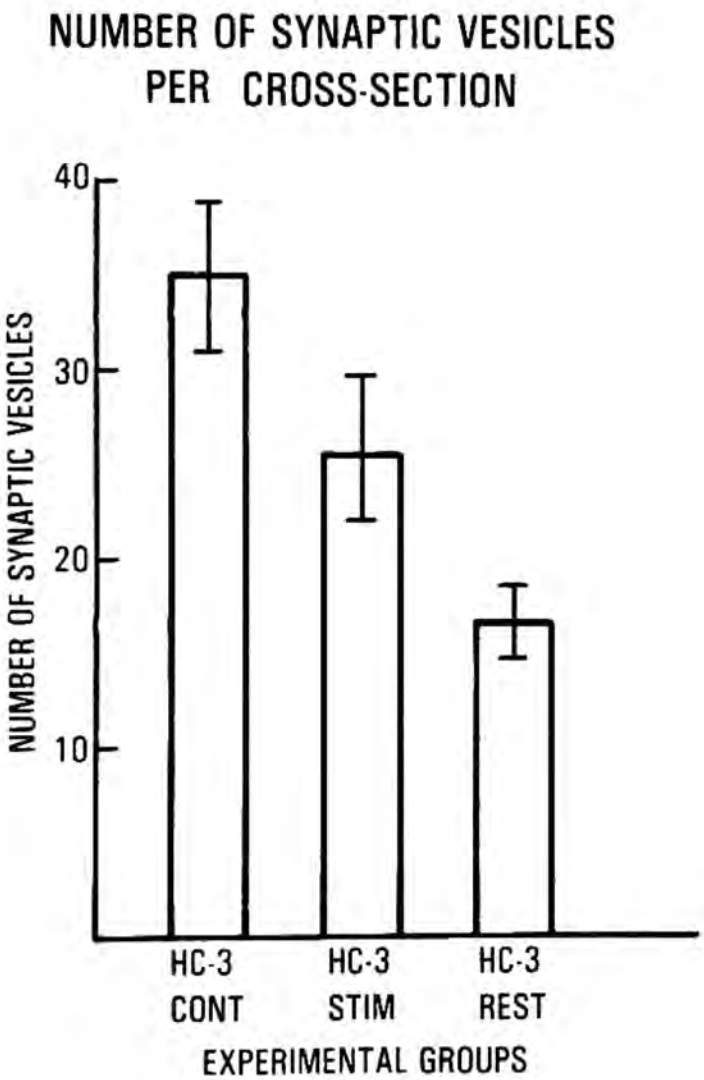
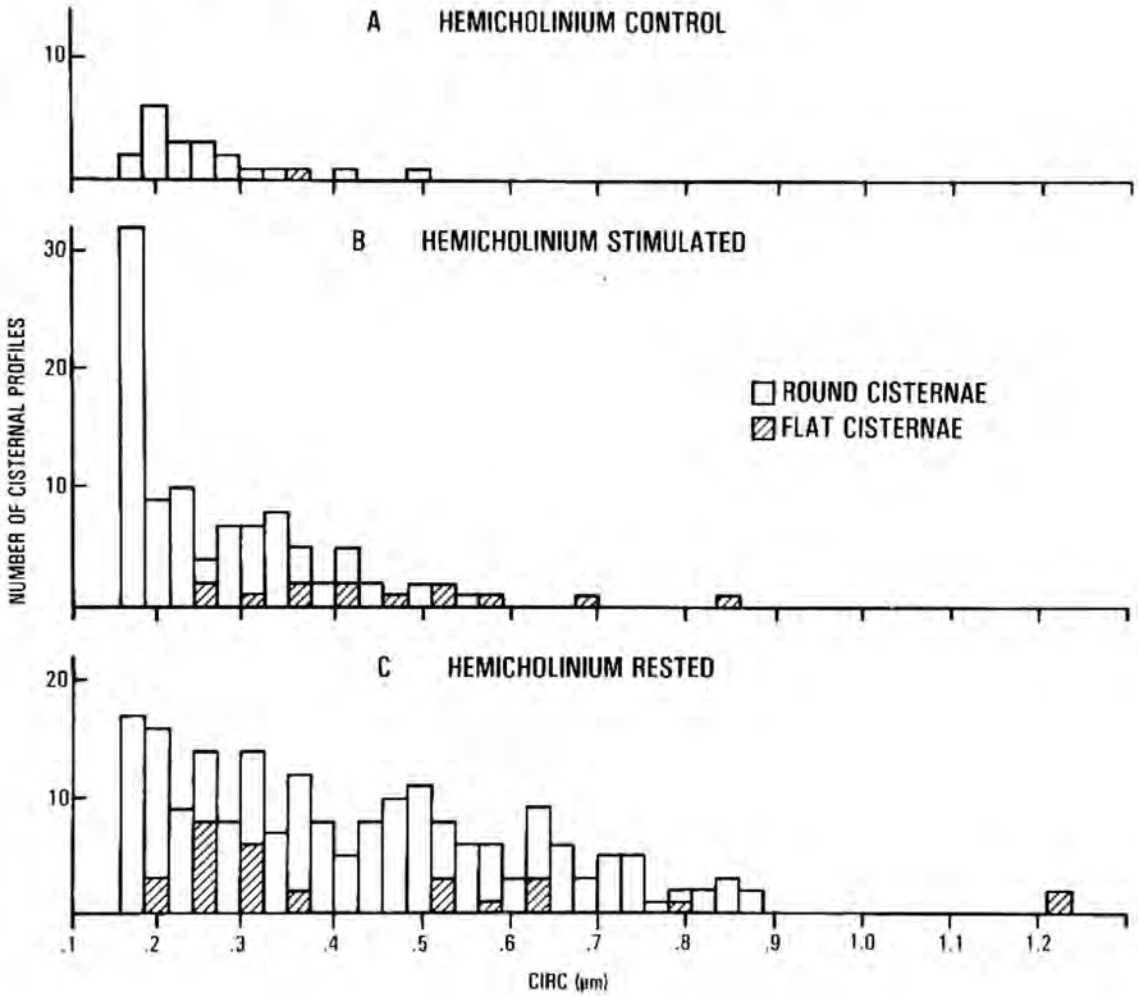

NUMBER OF SYNAPTIC VESICLES
PER CROSS-SECTION
NUMBER OF SYNAPTIC VESICLES
40
30
20
10
HC-3
CONT
HC-3
STIM
HC-3
REST
EXPERIMENTAL GROUPS

Figure 23. Histograms of the distribution of cisternal circumferences for the hemicholinium series.

These graphs contain all measurements from 15 micrographs from a representative muscle in each of the three groups in the hemicholinium series. A progressive increase in both number and size of cisternal profiles was seen with stimulation and subsequent rest in HC-3.

DISTRIBUTION OF CISTERNAL CIRCUMFERENCES



(23)

Figure 24. A histogram of total cisternal circumference per nerve terminal cross-section for the hemicholinium series.

Each bar represents the mean amount of cisternal membrane (±SE) per nerve terminal cross-section in each of the three experimental groups of the hemicholinium series. Each mean was calculated from the mean total cisternal circumference per nerve terminal cross-section for all three muscles in each experimental group. A progressive increase in the amount of cisternal membrane was observed after stimulation and subsequent rest in HC-3.

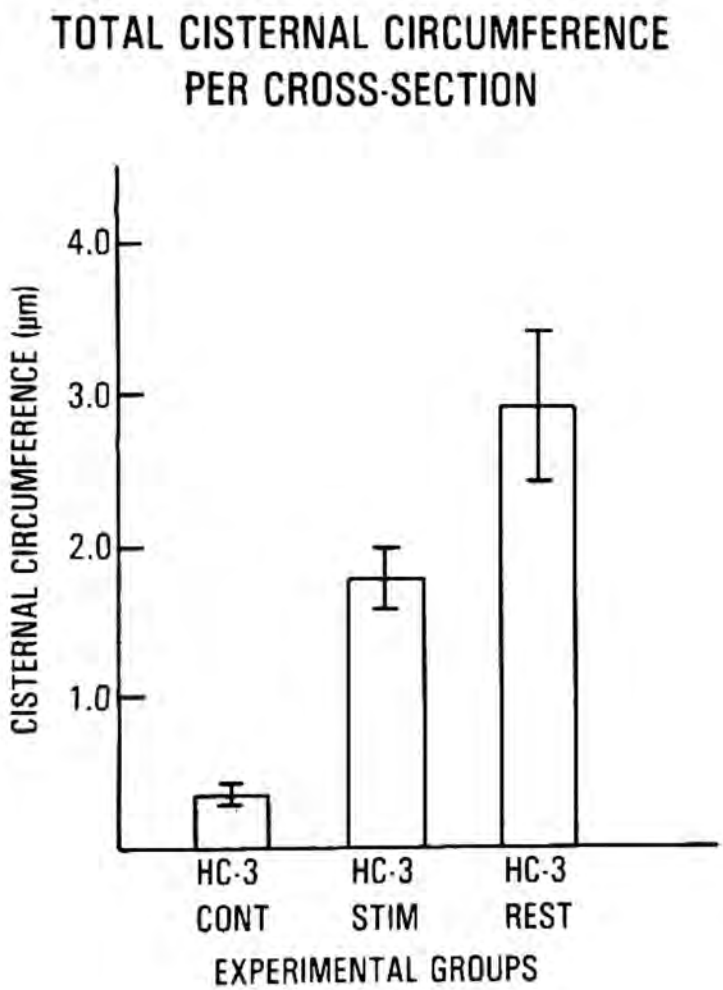

TOTAL CISTERNAL CIRCUMFERENCE
PER CROSS-SECTION
CISTERNAL CIRCUMFERENCE (μm)
4.0
3.0
2.0
1.0
HC-3
CONT
HC-3
STIM
HC-3
REST
EXPERIMENTAL GROUPS

Figure 25. A histogram of the number of mitochondrial profiles per nerve terminal cross-section for the hemicholinium series.

Each bar represents the mean number of mitochondrial profiles (±SE) per nerve terminal cross-section in each of the three groups in the hemicholinium series. Each mean was calculated from the mean number of mitochondrial profiles per nerve terminal cross-section for all three muscles in each experimental group. With stimulation the number of mitochondrial profiles per nerve terminal cross-section decreased from control counts. With rest the number of mitochondria remained the same as the stimulated group, indicating that the mitochondria did not undergo further shape changes.

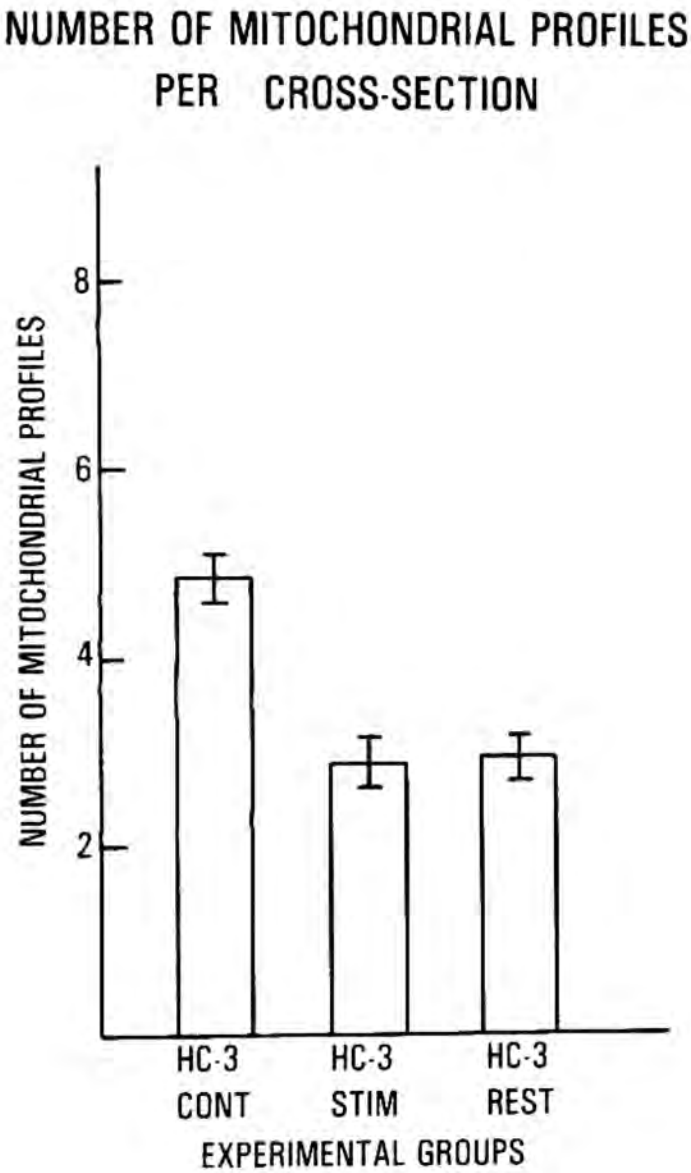
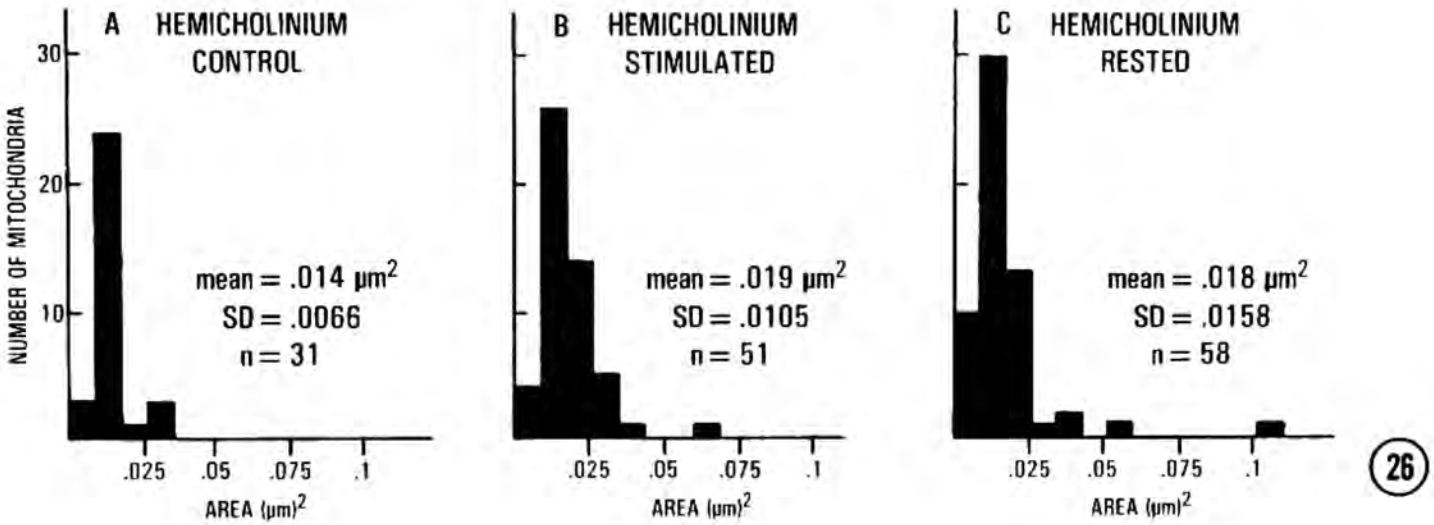

NUMBER OF MITOCHONDRIAL PROFILES
PER CROSS-SECTION
NUMBER OF MITOCHONDRIAL PROFILES
8
6
4
2
HC-3
CONT
HC-3
STIM
HC-3
REST
EXPERIMENTAL GROUPS

Figure 26. Histograms of the distribution of mitochondrial cross-sectional areas for the hemicholinium series.

Each histogram represents all measurements from 15 micrographs from a representative muscle in each of the three experimental groups in the hemicholinium series. Mitochondria in rapid-frozen hemicholinium control nerve terminals (A) were tightly clustered around an average area of .014 μm . With stimulation (B) mitochondrial area increased only slightly and the number of mitochondrial profiles per nerve terminal cross-section did not drop. With rest (C) mitochondria did not change from their stimulated appearance.

DISTRIBUTION OF MITOCHONDRIAL CROSS-SECTIONAL AREAS



26

DISCUSSION

I. Effects of fixation on presynaptic nerve terminals

It has been proposed that chemical fixatives, particularly aldehydes, can cause swelling and distortion of tissue (Lee, McKenzie, Kobayashi, Garfield, Forrest and Daniel, 1982). The results of the present study support this idea *and* indicate that rapid-freezing preserves tissues with fewer distortions and artifacts.

In general, the terminals, as well as most organelles, were larger in the chemically fixed nerve terminals than in the rapid-frozen terminals. This may be due to general swelling of tissues upon fixation, or to uniform shrinkage of tissue during freeze-substitution. Following rapid-freezing, tissue is placed in an acetone-OsO_4 mixture. Dehydration of the tissue may precede stabilization of structures by osmium. If shrinkage of frozen material is responsible for the size difference between fixed and frozen material, it should have produced uniform shrinkage of all structures. There are fairly uniform differences (about 25%) in the sizes of such structures as synaptic vesicles, DCVs and the terminals themselves in control fixed and frozen tissue. Superimposed on these differences were greater differences

in the sizes of some organelles. These latter differences may be attributable to interaction of the tissue with the chemical fixative.

Although both chemically fixed and rapid-frozen terminals exhibited changes after stimulation that were consistent with the vesicle recycling hypothesis, changes in chemically fixed NMJs were more extensive than changes in the frozen terminals. While both preparations had fewer synaptic vesicles after stimulation there was a concomitant increase in the amount of plasma membrane only in the fixed terminals. Fixed terminals were also swollen (increased in terminal cross-sectional area) after stimulation while the frozen terminals did not appear to be. This indicated that chemical fixation appeared to cause a swelling of the nerve terminal.

The two types of preservation resulted in different cisternal shapes. Flat cisternae comprised a larger percentage of the cisternal population in the stimulated fixed material while round cisternae were more prevalent than the flat ones in the frozen terminals. Analysis of serial sections in both fixed and frozen tissue revealed that round cisternae could coalesce to form larger flattened cisternae. This indicated that the shape differences did not necessarily reflect functionally or chemically different organelles.

With stimulation the number of cisternal profiles

increased more in the frozen terminals than in the fixed, whereas the average cisternal size greatly increased only in the fixed. The total cisternal membrane, however, increased by approximately the same amount, regardless of the mode of preservation. The difference in size and number of cisternae between stimulated terminals preserved by the two methods reveals a fixation artifact: The results obtained from the frozen material indicate that stimulation causes an increase in the number of small round cisternae. Subsequent fixation appears to induce fusion of the small cisternae to form larger, often flattened structures.

While aldehyde fixation causes fusion of cisternae, standard sized synaptic vesicles remain as an independent stable population. This finding suggests a difference between the contents or membrane composition of synaptic vesicles and cisternae. Flattening of synaptic vesicles has been attributed to fixation artifact in both inhibitory synapses and stimulated NMJs (Valdivia, 1971; Korneliussen, 1972b). Birks (1971) saw a stimulation-associated increase in the number of flattened synaptic vesicles in fixed frog NMJs. These results are supported by the present study. Flattened synaptic vesicles (found only in fixed terminals) did increase after stimulation and decrease with rest. Since the artifact is consistently associated with stimulation, it

must represent some real underlying change in vesicle contents or membrane.

Any change in synaptic vesicle diameter in the fixed terminals was slight and would require an impractical number of experiments to verify statistically. Chemical fixation may have masked the normal stimulation-associated increase in synaptic vesicle diameter by causing all organelles (including synaptic vesicles) to swell. Even with the stimulation-induced increases in their diameter, synaptic vesicles in frozen terminals were never as large as those in the chemically fixed terminals.

Many investigators have noted changes in morphology of presynaptic mitochondria with synaptic activity. Increases in the diameter of these organelles following stimulation has been commonly observed in chemically fixed nerve terminals (Jones and Kwanbunbumpen, 1970a; Parducz and Feher, 1970; Ceccarelli, Hurlbut and Mauro, 1973; Dickinson and Reese, 1973; Heuser and Reese, 1973; Model, Highstein and Bennett, 1975; but see Parducz and Joo, 1976). Mitochondria have also been observed to change positions in presynaptic terminals after stimulation, moving closer to the plasma membrane (Jones and Kwanbunbumpen, 1970a), or mingling with synaptic vesicles (Korneliussen, 1972a; Heuser and Reese, 1973).

A study by Hackenbrock and Caplan (1969) on

isolated liver mitochondria showed that these organelles swelled when they were exposed to a $CaPO^{-2}$ environment. In the absence of phosphate ions, it was shown that the mitochondria sequestered calcium, but that they did not swell. Subsequent exposure of these mitochondria to phosphate resulted in swelling. These results caused the authors to propose that it was the exposure to phosphate, not the calcium sequestration, that caused mitochondria to swell. This becomes significant when one realizes that the intracellular phosphate concentration may increase during fixation, leading to this morphological artifact.

Evidence from the present experiments also established that the stimulation-induced enlargement of mitochondria observed in chemically fixed tissue is largely an artifact of fixation. Rapid-freezing shows that stimulation alone causes a reduction in the number of mitochondrial profiles and an increase in their average diameter, without any change in the total mitochondrial volume (ie., cross-sectional area) per terminal. This was interpreted as evidence that mitochondria were shortening in length and increasing in diameter (rounding-up) without swelling (ie., increasing in volume). With rounding-up, mitochondrial profiles would be less frequent in any given cross-section and their average cross-sectional area would be greater. This interpretation of the results assumes that the absolute number of mitochondria in a nerve

terminal arborization does not change with stimulation. Since the functions of mitochondria relating to energy production and calcium sequestration are important during nerve activity it seems unlikely that these organelles would migrate from the terminal area with stimulation. In longitudinal sections of chemically fixed frog nerve terminals, mitochondria have been shown to be linear organelles that run parallel to the long axis of the terminal (Heuser and Reese, 1973; Ceccarelli, Hurlbut and Mauro, 1973). With this evidence, the hypothesis that a decreased number of mitochondrial profiles following stimulation could be a result of increased coiling or twisting of these organelles is not substantiated.

Assuming that the interpretation discussed above is correct, mitochondria in stimulated terminals preserved by chemical fixation shortened in length and swelled greatly, in contrast to the response revealed by rapid-freezing. With rest followed by chemical fixation, they elongated beyond control lengths but only partially decreased in volume.

II. Effects of stimulation and rest on presynaptic nerve terminals preserved by rapid-freezing

The primary purpose of these experiments was to

determine the morphological changes seen in rapidly stimulated NMJs preserved by rapid-freezing and freeze-substitution. The presynaptic effects of rapid stimulation (15 Hz for 15 min) as seen in the first set of experiments were: 1) a reduction in the number of synaptic vesicles; 2) an increase in synaptic vesicle diameter; 3) an increase in the number of cisternal profiles; 4) a change in the shape of mitochondria without any detectable change in their volume and 5) a decrease in the number of microtubules. With rest, synaptic vesicles and cisternae recovered close to control while other organelles differed in their return toward control values.

A. Balance of membrane in the presynaptic terminal

The Heuser-Reese (1973) theory of synaptic vesicle membrane recycling proposes that, following release of transmitter by the synaptic vesicles, their membrane is incorporated into the presynaptic plasma membrane. This process temporarily increases the amount of nerve terminal plasma membrane. Equal amounts of membrane are then recovered from the plasma membrane by the formation of coated vesicles or by direct invagination (Heuser, 1976). New synaptic vesicles then bud from cisternae, formed by direct invagination and/or fusion of coated vesicles.

This theory accounts for maintenance of the synaptic vesicle population and constancy of total membrane in the presynaptic nerve terminal during prolonged synaptic activity.

The results in rapid-frozen material in the present study are generally consistent with this recycling hypothesis. The number of synaptic vesicles diminished with stimulation. A concomitant rise was detected in the number of cisternal profiles. No increase in terminal plasmalemma was measured, however, and although coated vesicles increased slightly in number after stimulation there were so few that it was not possible to make definitive quantitative statements about them.

One reason for the scarcity of coated vesicles in the present experiments could be the time that elapsed between the cessation of stimulation and freezing. Miller and Heuser (1979) counted coated pits in rapid-frozen freeze-fractured frog NMJs stimulated in 4-aminopyridine (4-AP). They noted that the coated invaginations began to form 1 second after stimulation, peaked at 30 seconds, and disappeared almost completely by 90 seconds. Since the time elapsed between the end of stimulation and freezing in the current studies was 60 seconds, it seems reasonable that most coated structures had already been formed and disassembled by the time the cellular processes were arrested. This hypothesis may also account for the

finding that plasmalemma was not increased after stimulation, a phenomenon reported in several studies of chemically fixed material (Ceccarelli, Hurlbut and Mauro, 1972, 1973; Heuser and Reese, 1973; Pysh and Wiley, 1974). After a period of 60 seconds, any excess membrane added to the plasma membrane may already have been converted to cisternae.

To further investigate the relationship between the reduction in synaptic vesicle number and increased cisternae, experiments were conducted in which the ionic concentration of the extracellular bathing medium was altered. Since stimulation of the presynaptic nerve terminal causes an increase in intraterminal calcium, and this calcium mediates the mechanism whereby synaptic vesicles move to the active zone of the presynaptic membrane and fuse to release transmitter (for review see Kelly, Deutsch, Carlson and Wagner, 1979), it was predicted that increasing the concentration of calcium in the extracellular bathing medium would cause a greater reduction in the number of synaptic vesicles. Also, since magnesium is antagonistic to calcium at the nerve terminal, stimulation in high magnesium Ringer's solution should produce no changes in terminal morphology.

Soaking the terminals in either high calcium or high magnesium Ringer's solution caused a slight decrease

in the number of synaptic vesicles, with respect to controls in normal Ringer's solution. There were few or no concomitant changes in the numbers of cisternal profiles. Terminals stimulated in high calcium Ringer's solution showed further, profound reduction of synaptic vesicles and a greatly increased population of round cisternae. Without biochemical markers it is not possible to determine the exact source of the membrane in these structures but several possibilties exist. Increased calcium may have increased the rate of transmitter release, causing membrane to accumulate in the cisternal form. The severe reduction in synaptic vesicle number in terminals stimulated in high calcium Ringer's solution supports this interpretation. However, it is also possible that vesicular structures are involved in calcium sequestration and contribute to the cisternal population during the sequestration process. (See Discussion II-B for further discussion of this point.)

In the present study, terminals soaked in high magnesium Ringer's solution and preserved by rapid-freezing did show some morphological changes from normal controls. Synaptic vesicle numbers were reduced by soaking in magnesium. There was additional loss (15%) due to stimulation in high magnesium Ringer's solution. This additional loss may have been due to the displacement of residual bound calcium (Elmqvist and Feldman, 1966)

causing increased spontaneous release of neurotransmitter. The number of cisternal profiles, while lower in magnesium control terminals than in normal controls, decreased even more after stimulation in magnesium Ringer's.

Heuser, Katz and Miledi (1971) found that isotonic $MgCl_2$ caused a transient increase in MEPP frequency. By qualitative evaluation, their terminals demonstrated normal ultrastructure except for an apparent increase in the number of cisternae, and synaptic vesicles which bulged out against the presynaptic membrane. Synaptic vesicle concentrations were not quantitatively assessed.

In the magnesium stimulated terminals, sodium, which enters during an action potential, may facilitate ACH release by producing a displacement of intracellular bound calcium, (Birks, 1963; Charlton and Atwood, 1977; Erulkar and Rahamimoff, 1978; Misler and Hurlbut, 1983). This would be consistent with the reduction in vesicle numbers observed. One would expect cisternae to increase to balance vesicle reduction, however, and this did not occur. The "lost" synaptic vesicle membrane can be accounted for only by the increase in synaptic vesicle size, to the largest size of any rapid-frozen experimental group.

In the present study, terminals were soaked in Ringer's solution containing 100 μM HC-3 Ringer's to

determine if synaptic vesicle reloading plays a role in the stimulation-induced changes in vesicle number. These terminals had fewer synaptic vesicles than control terminals in normal Ringer's. Following stimulation in HC-3 the number of synaptic vesicles decreased further and large increases were noted in the number of cisternal profiles. A 60 minute period of rest in HC-3 exacerbated these results, causing further reduction of synaptic vesicles and continued increase in cisternae. These terminals thus gave every indication of stimulation just by being soaked in HC-3. Stimulation exaggerated these effects and no recovery was seen.

In the presence of HC-3, high frequency stimulation of rat NMJs causes a reversible decrease in quantal size of both EPPs and MEPPs, whereas in the absence of stimulation no change in quantal size is seen (Elmqvist, Quastel and Thesleff, 1963). These results suggested that HC-3 reduced the amount of ACH loaded into recycled synaptic vesicles but, without stimulation had no effect on the synaptic vesicles.

Reports of the effect of HC-3 on synaptic vesicle number have produced conflicting evidence. Ceccarelli and Hurlbut (1975) reported an irreversible depletion of ACH when frog NMJs were stimulated in HC-3 (measured electrophysiologically). They saw no depletion of synaptic vesicle numbers, however, and suggested that the

synthesis of ACH and the recycling of synaptic vesicle membrane are independent processes. In contrast, Parducz, Kiss and Joo (1976) observed a decrease in synaptic vesicle number in cat superior cervical ganglion after stimulation in HC-3. Their explanation was that ACH synthesis for neurotransmission exerts such a great demand on the intraterminal choline pool that when high affinity choline uptake is inhibited by HC-3, synaptic vesicle membrane may be degraded into an ACH precursor, phosphatidylcholine.

Results from the current study could be due to: 1) HC-3 causing a depolarization of the terminal 2) toxic effects of HC-3, perhaps causing plasma membrane disruption and subsequent changes due to calcium leakage into the terminal 3) HC-3 used containing a contaminant.

B. Stimulation-induced swelling of synaptic vesicles in rapid-frozen nerve terminals

The results of the current study agree with previous reports of increased vesicle size following stimulation (Heuser and Reese, 1973; Fried and Blaustein, 1978; Lynch, 1982). Average synaptic vesicle diameter in the present experiments increased following stimulation

and returned to control range following a period of rest. Although synaptic vesicles were defined in a new way in the present study, it is unlikely that the definition procedure totally accounts for the differences in synaptic vesicle diameter.

As stated in Materials and Methods, all vesicular profiles were measured on enough micrographs per muscle to allow plotting of a histogram. Based on the peak of the single normal curve observed on these histograms, cutoff points were individually determined between synaptic vesicles and cisternae. Profiles below this cutoff were defined as synaptic vesicles, profiles above as round cisternae. This procedure provided a somewhat arbitrary distinction between structures that differ in a two-dimensional micrograph only in their size. Because the smaller structures yielded a normal curve in each of the histograms, however, it was felt that they represented a distinct population of structures, ie. "synaptic vesicles". The structures above the cutoff, collectively called "cisternae" were of variable diameters and their profiles, therefore, were not normally distributed. Additional evidence for two distinctive populations of membranous organelles comes from fixed experimental groups (see Discussion I). The increase in synaptic vesicle diameter with stimulation may have been due, at least in part, to increases in the number of small cisternae.

Off-center sections through these structures would create some synaptic vesicle-sized profiles and might skew the mean synaptic vesicle diameter upward. In at least one set of experiments, however, the magnesium stimulated preparations, synaptic vesicles increased in size without a concomitant increase in the number of cisternae. After stimulation, all of the synaptic vesicles were larger, leaving no structures of the same size as the magnesium control synaptic vesicles. In addition, the mean synaptic vesicle diameter in calcium stimulated terminals did not increase more than in those stimulated in normal Ringer's, even though the number of small cisternal profiles was more than twice as great. These findings were interpreted as evidence that the shift in synaptic vesicle size seen in other experimental groups was real and not due entirely to an addition of small cisternal profiles.

Vesicle enlargement may be the result of a change in osmotic properties of the vesicle contents, in the amount or composition of the vesicle contents, or the properties of the vesicle membrane and the concentration of pumps within it. Return of vesicle diameter to normal with rest shows that degeneration of nerve terminals is not responsible for the changes seen in the present study. Perhaps when vesicles are initially reformed they contain extracellular fluid which would cause them to be slightly enlarged. With rest, this fluid may be pumped

out of the vesicles or otherwise lost. The lack of bimodality in the synaptic vesicle diameters suggests that all of the synaptic vesicles are larger, not just a subpopulation that could be interpreted as recycled ones. All the synaptic vesicles in a terminal could be turned over within 15 minutes of stimulation (Ceccarelli, Hurlbut and Mauro, 1973; Heuser and Reese, 1973). Those measured in stimulated preparations could therefore be only recycled synaptic vesicles.

Another possible explanation for the uniform increase in diameter is that the increase in synaptic vesicle diameter seen after stimulation is related to their reloading with ACH (Large and Rang, 1978a, 1978b). If vesicle reloading were responsible for synaptic vesicle diameter increase, HC-3 should have blocked the increase. Vesicle size might even be expected to decrease because the vesicles would be unable to refill with transmitter. In the present study, nerve terminals soaked, stimulated or rested in HC-3 Ringer's showed many changes but a decrease in synaptic vesicle diameter was not one of them. In fact the synaptic vesicles in HC-3 soaked terminals were slightly larger than those in terminals soaked in normal Ringer's.

Some authors feel that synaptic vesicles serve as calcium sequestering organelles (Bohan, Boyne, Guth, Narayan and Williams, 1973; Politoff, Rose and Pappas,

1974; Israel, Manaranche, Marsal, Meunier, Morel, Franchon and Lesbats, 1980). It seems unlikely that the swelling observed with stimulation could result from sequestration of an increased intracellular calcium load. A greater increase in diameter would have been expected after stimulation in high calcium Ringer's, while no increase should have been observed after stimulation in high magnesium Ringer's solution. In the present experiments, soaking the nerve-muscle preparation in either high calcium or high magnesium Ringer's solution did not produce any detectable changes in the synaptic vesicle diameter. While increases in synaptic vesicle diameter following stimulation were noted in both groups, it was no greater in calcium than in normal Ringer's and was most pronounced in the magnesium stimulated terminals

C. Mitochondrial shape change following stimulation

It has been observed in stimulated chemically fixed terminals that the number of mitochondrial profiles per unit length of terminal decreased after stimulation (Lynch, 1982). While this might indicate a mass migration of mitochondria from the presynaptic terminals, it is unlikely since their presence is required both for calcium

sequestration and energy production, especially when synaptic activity is high. It is more likely that a change in shape, a rounding-up, changed the frequency with which mitochondria appeared in the average terminal cross-section.

Results from the present experiments support the hypothesis that a shape change occurs in mitochondria when nerve terminals are stimulated. This shape change apparently involves a shortening of the organelles, seen as a reduction in mitochondrial profiles following stimulation accompanied by an increase in mitochondrial cross-sectional area. Total mitochondrial area per terminal cross-section remained constant, indicating that there was no increase in the absolute volume of mitochondria, ie. no swelling.

Mitochondria have been shown to be calcium-sequestering organelles (Lehninger, 1970; Rahamimoff, 1976). This calcium sequestration has been linked to mitochondrial enlargement in fixed tissue. Therefore, shape changes observed in frozen tissue might increase after stimulation in high calcium Ringer's. While an indication of the expected stimulation-induced shape change was seen, the sample sizes were too small to say whether it is the calcium sequestering function of mitochondria that causes them to change shape after stimulation. The stimulation-induced shape changes in

mitochondria in calcium-soaked terminals were not different from those in terminals soaked and stimulated in normal Ringer's solution. There was some indication, however, that the mitochondria swelled slightly with stimulation in high calcium, a change that was not seen in the first set of experiments. Swelling with stimulation was also suggested by the results of the normal Ringer's experiments in the second series and was also observed in the HC-3 experiments. With rest after stimulation, the number of mitochondrial profiles remained reduced and their mean diameter, though reduced, was still higher than before stimulation. Total mitochondrial volume per terminal decreased, however, indicating either that mitochondria contracted or migrated from the terminals.

Electron-dense deposits have been observed in mitochondria in chemically fixed, stimulated terminals (Heuser and Reese, 1973). These deposits are similar to those seen in mitochondria which have actively sequestered calcium (Greenawalt, Rossi and Lehninger, 1964; Peachy, 1964; Lehninger, 1970;) and are often present when special solutions are used, presumably trapping calcium in an insoluble form (Becker, Canada and Pappas, 1982; Phillips and Boyne, 1982).

No special solutions were used in the present study but some deposits were noted in mitochondria. They were infrequent and no pattern to their presence was

noted, with two exceptions. Granules were consistently present in mitochondria of presynaptic terminals and postsynaptic muscle in the preparations stimulated in HC-3. Many of these mitochondria demonstrated holes or electron-dense granules. Their relationship to calcium sequestration is unknown. Electron-dense granules were also occasionally seen in the mitochondria of nerve terminals that had been stimulated in high calcium Ringer's solution. These results support a recent study by Hirokawa, Heuser and Evans (1981) in both rapid-frozen and chemically fixed frog NMJs. These workers demonstrated the presence of granules after stimulation (25 Hz for 5 min) in high calcium (10 mM) Ringer's. They found that granules were also present when terminals were stimulated in normal Ringer's and freeze-substituted with 5% OsO_4/25 mM oxalic acid. They could increase both the number of granules and mitochondrial diameter when they stimulated terminals in 10 mM Ca Ringer's. They observed no granules in either unstimulated terminals in high calcium Ringer's or in terminals stimulated in 10 mM Mg Ringer's.

D. Effects of stimulation on other presynaptic organelles

1. Microtubules (MTs)

A previously unreported effect of stimulation on nerve terminals was a decrease in the number of MTs which did not recover with rest. Disassembly of cytoskeleton by calcium-activated cytoplasmic proteases and other mechanisms has been proposed (Lasek and Black, 1977; Schlaepfer, Zimmerman and Micko, 1981). It is possible that nerve stimulation may induce the breakdown of MTs by these enzymes by increasing intracellular calcium.

Results from experiments in the second set of experiments did not support the above findings. The number of MTs per terminal cross-section in control muscles was extremely low and, after stimulation, increased to levels equal to control levels in the first set of experiments. These differences may be related to differences in the age, developmental stage, seasonal state or activity level of animals used in the two sets of experiments. Changes in the number of microtubules with stimulation in the second set of experiments was consistently opposite to changes seen in the first set of experiments.

2. SER

A stimulation-induced decrease in the amount of SER identifiable as narrow-calibre, branched tubes was reported by Lynch (1982) in a study of chemically fixed terminals and Lynch hypothesized that the increased intracellular calcium following stimulation might be sequestered by the SER, causing it to swell. The SER has been shown to be a calcium-sequestering organelle in synaptosomes and squid axons (Henkart, 1975; Blaustein, Ratzleff and Schweitzer, 1978). In calcium-loaded squid axons, Henkart (1975) observed a shrinkage in SER following depolarization. This was thought to be linked to a depolarization-coupled calcium release.

One of the original aims of these experiments was to follow the effect of stimulation and recovery on SER in rapid-frozen terminals, however SER was not measurable in the current study. Its presence was noted in terminals of all experimental groups but a careful quantitative study was considered impossible because of the interference with its identification by preserved presynaptic matrix. Numerous freeze-substitution protocols were tested in preliminary experiments to improve reliable preservation of the SER but compromises had to be made to optimize preservation of other membranes.

3. DCVs

The number of DCVs present in the current study was too low to provide conclusive evidence, but they appeared to decrease with stimulation, without recovering in the succeeding hour. This is in accordance with the observations of Lynch (1980). If DCVs contain a peptide modulator of transmission (ChanPalay, Engel, Wu and Palay, 1982), the decrease seen may reflect the release of this substance during synaptic activity. Recycling of a peptide modulator would not be expected because such substances are produced in the neuronal soma and provided to the nerve ending by anterograde axonal transport.

III. Future considerations

Increased osmotic pressure has been postulated to cause a release of transmitter by a calcium-independent manner, also independent of nerve terminal depolarization (Smith and Reese, 1980). The initial transient effects of changes of osmotic gradient upon transmitter release are related to the flow of water through the terminal membrane. Later effects are related to volume changes of the terminal (Hubbard, Jones and Landau, 1968). These effects of increased osmotic pressure could be reflected

in a decreased number of synaptic vesicles in terminals soaked in a hyperosmotic solution and preserved by rapid-freezing.

A study by Clark (1976) involved soaking frog nerve terminals in 250 mM sucrose Ringer's solution for varying periods of time. Terminals soaked and immediately fixed were observed to have normal morphology, except for a slight increase in pleomorphic vesicles. Following periods of soaking ranging from 5 minutes to 2 hours, more vesicles became pleomorphic and increased in diameter 2-3 times control. Clumping of these synaptic vesicles caused them to push toward the plasma membrane and bulge it outward. To check the validity of these findings, Clark's experiments must be repeated in rapid-frozen material.

If synaptic vesicle swelling after stimulation (as seen in the rapid-frozen terminals in the present study) is due to an osmotic imbalance, an increased osmolarity of Ringer's solution could be reflected in increased synaptic vesicle diameter. In a Ringer's solution made hypertonic by the addition of sucrose, synaptic vesicles may increase in size by endocytosing this extracellular sucrose during membrane recycling. The synaptic vesicles would be unable to pump sucrose out with rest and thus would show no recovery. Study of this hypothesis as well requires preserving tissue by rapid-freezing.

IV. Conclusions

In conclusion, the results of the present study have confirmed much of the work of previous investigators in chemically fixed material regarding the morphological changes seen in presynaptic nerve terminals following stimulation. New observations made in rapid-frozen material demonstrated that some of the previously observed presynaptic changes may be artifacts of fixation (eg. mitochondrial swelling and increases in cisternal size) and that chemical fixation may have masked other changes (eg. increased synaptic vesicle diameter). A new observation made in both rapid-frozen and chemically fixed terminals was the reduction of microtubule numbers after stimulation in the first set of experiments. Observations made in the second set of experiments did not support this finding and raised the possibility that the response of the cytoskeleton to stimulation may differ with seasonal or functional states of the animal.

Although rapid-freezing is a relatively new preservation technique, it is being adopted with increasing frequency by numerous investigators. The results of the present study demonstrate that rapid-freezing presents a valid alternative to chemical fixation, and that, in many ways, is preferable because of the elimination of fixation artifacts.

APPENDIX

I. Statistics

A. Means and variances were calculated for each of 28 variables in each of the muscles per experimental group. Variables were:

1. terminal area
2. terminal circumference
3. number of round synaptic vesicles
4. number of flat synaptic vesicles
5. number of coated pits
6. number of coated vesicles
7. total coated vesicle circumference
8. number of dense-cored vesicles
9. total dense-cored vesicle circumference
10. number of round cisternae
11. total round cisternal circumference
12. number of flat cisternae
13. total flat cisternal circumference
14. number of mitochondria
15. total mitochondrial area
16. total mitochondria circumference
17. number of microtubules

18. total neurofilament area
19. total number of synaptic vesicles
20. total number of cisternae
21. total cisternal circumference
22. average coated vesicle circumference
23. average dense-cored vesicle circumference
24. average round cisternal circumference
25. average flat cisternal circumference
26. average mitochondrial area
27. average mitochondrial circumference
28. average round synaptic vesicle diameter

B. Comparisons were made between the following pairs of experimental groups:

1. First set of experiments

    a. Fixed control and fixed stimulated
    b. Fixed control and fixed rested
    c. Frozen control and frozen stimulated
    d. Frozen control and frozen rested
    e. Fixed control and frozen control
    f. Fixed stimulated and frozen stimulated
    g. Fixed rested and frozen rested

2. Calcium/magnesium set of experiments

a. Normal control and normal stimulated
b. Calcium control and normal control
c. Magnesium control and normal control
d. Calcium control and calcium stimulated
e. Magnesium control and magnesium stimulated

C. Hemicholinium set of experiments

a. Hemicholinium control and normal control
b. Hemicholinium control and hemicholinium stimulated
c. Hemicholinium control and hemicholinium rested

VI. Solutions

A. Normal Ringer's (250 mosm)

111.0 mM	NaCl
2.0	KCl
1.8	$CaCl_2$
5.0	HEPES
3.0	Dextrose

B. Calcium Ringer's (250 mosm)

98.7 mM	NaCl
2.0	KCl
10.0	$CaCl_2$
5.0	HEPES
3.0	Dextrose

C. Magnesium Ringer's (205 mosm)

98.7 mM	NaCl
2.0	KCl
10.0	$MgCl_2$
5.0	HEPES
3.0	Dextrose

D. Hemicholinium-3 Ringer's (226 mosm)

100.0 μM HC-3 in normal Ringer's (A)

E. Curare Ringer's (250 mosm)

.75 μg/ml in normal Ringer's (A)

F. Cacodylate buffer

90.0 mM sodium cacodylate

20.0 mM $CaCl_2(2H_2O)$

G. Primary fixative

3% glutaraldehyde in normal Ringer's (A)

H. Secondary fixative

1.0% OsO_4

1.5% potassium ferrocyanide

in half-strength cacodylate buffer

I. Freeze-substitution medium

4% OsO_4 in acetone

J. Block stain

10% uranyl acetate in methanol

K. Uranyl acetate grid stain

10% uranyl aetate in 45% methanol

L. Lead citrate grid stain

2.7% lead nitrate

3.5% sodium citrate($2H_2O$)

26.7% NaOH

73.3% distilled water

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